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BALLANTINE, DR. AND MRS. H. T., JR.
BANG, MRS. FREDERIK B.
BANKS, MR. AND MRS. W. L.
BARROWS, MRS. ALBERT W.
BENNETT, DR. AND MRS. MICHAEL V. L.
BERNHEIMER, DR. ALAN W.
BERNSTEIN, MR. AND MRS. NORMAN
BIGELOW, MRS. ROBERT O.
BLACKBURN, DR. AND MRS. GEORGE L.
BODEEN, MR. AND MRS. GEORGE H.
BOETTIGER, DR. AND MRS. EDWARD G.
BOLTON, MR. AND MRS. THOMAS C.
BORGESE, DR. AND MRS. THOMAS A.
BOTKIN, DR. DANIEL B.
BOWLES, DR. AND MRS. FRANCIS P.
BRADLEY, DR. AND MRS. CHARLES C.
BRONSON, MRS. SAMUEL C.
BROWN, MRS. DUGALD E. S.
BROWN, DR. AND MRS. F. A., JR.
BROWN, DR. AND MRS. THORNTON
BUCK, MRS. JOHN B.
BUFFINGTON, MRS. ALICE H.
BUFFINGTON, MRS. GEORGE
BURGER, DR. AND MRS. MAX M.
BURROUGH, MRS. ARNOLD H.
BURT, MR. AND MRS. CHARLES E.
BUTLER, MRS. E. G.
BUTLER, MR. AND MRS. RHETT W.
CALKINS, MR. AND MRS. G. N., JR.
CAMPBELL, DR. AND MRS. DAVID G.
CAMPBELL, MR. AND MRS. WORTHINGTON, JR.
CAPOBIANCO, MR. AND MRS. PAT J.
CARLSON, DR. AND MRS. FRANCIS
CARLTON, MR. AND MRS. WINSLOW G.
CASHMAN, MR. AND MRS. EUGENE R.

Chambers, Dr. and Mrs. Edward L.
Cheney, Dr. and Mrs. Ralph H.
Claff, Mr. and Mrs. Mark
Clark, Mr. and Mrs. Hays
Clark, Mrs. James McC.
Clark, Dr. and Mrs. Leonard B.
Clark, Mr. and Mrs. LeRoy, Jr.
Clark, Mrs. W. Van Alan
Clement, Dr. and Mrs. A. C.
Clowes Fund, Inc.
Clowes, Dr. and Mrs. Alexander W.
Clowes, Mr. Allen W.
Clowes, Dr. and Mrs. G. H. A., Jr.
Cohen, Dr. and Mrs. Seymour
Coleman, Dr. and Mrs. John
Connell, Mr. and Mrs. W. J.
Cooper, Mr. and Mrs. John H., Jr.
Copeland, Mrs. D. Eugene
Copeland, Mr. and Mrs. Preston S.
Costello, Mrs. Donald P.
Crain, Mr. and Mrs. Melvin C.
Cramer, Mr. and Mrs. Ian D. W.
Crane, Mr. John
Crane, Josephine, Foundation
Crane, Mrs. W. Carey
Cross, Mr. and Mrs. Norman C.
Crossley, Mr. and Mrs. Archibald M.
Crowell, Dr. and Mrs. Sears
Daignault, Mr. and Mrs. A. T.
Daniels, Mr. and Mrs. Bruce G.
Day, Mr. and Mrs. Pomeroy
Dickson, Dr. William A.
Drummond, Mr. and Mrs. A. H., Jr.
DuBois, Dr. and Mrs. A. B.
Dunkerley, Mr. and Mrs. H. Gordon
DuPont, Mr. A. Felix, Jr.
Dyer, Mr. and Mrs. Arnold W.
Ebert, Dr. and Mrs. James D.
Edwards, Dr. and Mrs. Robert L.
Egloff, Dr. and Mrs. F. R. L.
Elliott, Mrs. Alfred M.
Elsmith, Mrs. Dorothy O.
Eppel, Mr. and Mrs. Dudley
Evans, Mr. and Mrs. Dudley
Ewing, Dr. and Mrs. Gifford C.
Fenno, Mrs. Edward N.
Ferguson, Dr. and Mrs. J. J., Jr.
Fine, Dr. and Mrs. Jacob
Fisher, Mrs. B. C.
Fisher, Mr. Frederick S., III
Fisher, Dr. and Mrs. Saul H.
Francis, Mr. and Mrs. Lewis W., Jr.
Fries, Dr. and Mrs. E. F. B.
Fye, Dr. and Mrs. Paul M.
Gabriel, Dr. and Mrs. Mordecai L.
Gaiser, Dr. and Mrs. David W.
Garfield, Miss Eleanor
Garrey, Dr. and Mrs. Walter
Gellis, Dr. and Mrs. Sydney
German, Dr. and Mrs. James L., III
Gifford, Mr. and Mrs. John A.
Gifford, Dr. and Mrs. Prosser
Gilbert, Dr. and Mrs. Daniel L.
Gilbert, Mrs. Helen H.
Gildea, Dr. Margaret C. L.
Gillette, Mr. and Mrs. Robert S.
Glass, Dr. and Mrs. H. Bentley
Glazebrook, Mrs. James R.
Glusman, Dr. and Mrs. Murray
Goldman, Dr. and Mrs. Allen S.
Goldstein, Mrs. Moise H., Jr.
Grant, Dr. and Mrs. Philip
Grassle, Mr. and Mrs. J. K.
Green, Miss Gladys M.
Greene, Mr. and Mrs. William C.
Greer, Mr. and Mrs. W. H., Jr.
Grosch, Dr. and Mrs. Daniel S.
Gross, Mrs. Paul R.
Gruson, Mrs. Martha C.
Gunning, Mr. and Mrs. Robert
Haigh, Mr. and Mrs. Richard H.
Halvorson, Dr. and Mrs. Harlyn O.
Handler, Mrs. Philip
Harvey, Dr. and Mrs. Richard B.
Hassett, Dr. and Mrs. Charles
Hastings, Mrs. J. Woodland
Heffron, Dr. and Mrs. Roderick
Henley, Dr. Catherine
Hiam, Mr. and Mrs. E. W.
Hiatt, Dr. and Mrs. Howard
Hill, Mrs. Samuel E.
Hilsinger, Mr. and Mrs. Arthur
Hirschfeld, Mrs. Nathan B.
Hobbie, Dr. and Mrs. John
Hocker, Mr. and Mrs. Lon
Hoffman, Rev. and Mrs. Charles
Horwitz, Dr. and Mrs. Norman H.
Houston, Mr. and Mrs. Howard E.
Huettner, Dr. and Mrs. Robert
Hunziker, Mr. and Mrs. Herbert E.
Hynes, Mr. and Mrs. Thomas J., Jr.
Inoué, Mrs. Shinya
Ireland, Mrs. Herbert A.
Issokson, Mr. and Mrs. Israel
Ivens, Dr. Sue
Jackson, Miss Elizabeth B.
Janney, Mrs. Wistar
Jewett, G. F., Foundation
Jewett, Mr. and Mrs. G. F., Jr.
Jones, Mr. and Mrs. DeWitt, III
Jones, Mr. and Mrs. Frederick, III
Jordan, Dr. and Mrs. Edwin P.
Kaan, Dr. Helen W.
Kahler, Mr. and Mrs. George A.

Kahler, Mr. and Mrs. Robert W.
Kaminer, Dr. and Mrs. Benjamin
Karush, Dr. and Mrs. Fred
Keith, Mrs. Jean R.
Kelleher, Mr. and Mrs. Paul R.
Keosian, Mrs. Jessie
Kien, Mr. and Mrs. Pieter
Kinnard, Mrs. L. Richard
Kivy, Dr. and Mrs. Peter
Kohn, Dr. and Mrs. Henry I.
Koller, Dr. and Mrs. Lewis R.
Kuffler, Mrs. Stephen W.
Laderman, Mr. and Mrs. Ezra
Lash, Dr. and Mrs. James
Laster, Dr. and Mrs. Leonard
Laufer, Dr. and Mrs. Hans
LaVigne, Margaret M.
Lawrence, Mr. Frederick V.
Lawrence, Mrs. William
Lazarow, Mrs. Arnold
Leatherbee, Mr. John A.
Lemann, Mrs. Lucy B.
Lenher, Mr. and Mrs. Samuel
Levine, Dr. and Mrs. Rachmiel
Lewis, Mr. John T.
Little, Mrs. Elbert
Loeb, Mrs. Robert F.
Lovell, Mr. and Mrs. Hollis R.
Lowe, Dr. and Mrs. Charles W.
Lowengard, Mrs. Joseph
Mackey, Mr. and Mrs. William K.
MacLeish, Mrs. Margaret
MacNary, Mr. B. Glenn
MacNichol, Dr. and Mrs. Edward F., Jr.
Maher, Miss Anne Camille
Marks, Dr. and Mrs. Paul A.
Marsland, Dr. and Mrs. Douglas
Martyna, Mr. and Mrs. Joseph
Marvin, Dr. Dorothy H.
Maser, Dr. and Mrs. Morton
Mastroianni, Dr. and Mrs. L., Jr.
Mather, Mr. and Mrs. Frank J., III
Matthiessen, Mr. and Mrs. G. C.
McCusker, Mr. and Mrs. Paul T.
McElroy, Mrs. Nella W.
McLane, Mrs. T. Thorne
Meigs, Mr. and Mrs. Arthur
Meigs, Dr. and Mrs. J. Wister
Melillo, Dr. and Mrs. Jerry
Mellon, Richard King, Foundation
Mellon, Mr. and Mrs. Richard P.
Menke, Dr. W. J.
Metz, Mrs. Charles B.
Meyers, Mr. and Mrs. Richard
Miller, Dr. Daniel A.
Mixter, Mr. and Mrs. W. J., Jr.
Montgomery, Dr. and Mrs. Charles H.
Montgomery, Mr. and Mrs. Raymond P.
Morse, Mr. and Mrs. Charles L., Jr.
Morse, Mr. and Mrs. Richard S.
Moul, Mrs. Edwin T.
Newton, C. H., Builders, Inc.
Nichols, Mrs. George
Nickerson, Mr. and Mrs. Frank L.
Norman, Mr. and Mrs. Andrew E.
Norman Foundation
O'Herron, Mr. and Mrs. Jonathan
O'Sullivan, Dr. Renee Bennett
Ortins, Mr. Armand
Palmer, Mrs. Douglas W.
Pappas, Dr. and Mrs. George D.
Park, Mrs. Franklin A.
Park, Mr. and Mrs. Malcolm S.
Parmenter, Miss Carolyn L.
Parmentier, Mr. George L.
Pendelton, Dr. and Mrs. Murray E.
Pendergast, Mrs. Claudia
Pennington, Miss Anne H.
Perkins, Mr. and Mrs. Courtland D.
Person, Dr. and Mrs. Philip
Peterson, Mr. and Mrs. E. Gunnar
Peterson, Mr. and Mrs. E. Joel
Peterson, Mr. Raymond W.
Philippe, Mr. and Mrs. Pierre
Porter, Dr. and Mrs. Keith R.
Prosser, Mrs. C. Ladd
Putnam, Mr. Allan Ray
Putnam, Mr. and Mrs. W. A., III
Pyne, Miss Ruth
Raymond, Dr. and Mrs. Samuel
Read, Ms. Lee
Redfield, Dr. and Mrs. Alfred C.
Renek, Mr. and Mrs. Morris
Reynolds, Dr. and Mrs. George
Reynolds, Mrs. Bartow
Reznikoff, Dr. and Mrs. Paul
Ricca, Dr. and Mrs. Renato A.
Riggs, Mr. and Mrs. Lawrasson, III
Riina, Mr. and Mrs. John R.
Robb, Ms. Alison A.
Robertson, Mrs. C. Stuart
Robertson, Dr. and Mrs. C. W.
Robinson, Dr. and Mrs. Denis M.
Rogers, Mrs. Julian
Root, Mrs. Walter S.
Ross, Dr. Virginia
Rowe, Mrs. William S.
Rubin, Dr. Joseph
Rugh, Mrs. Roberts
Russell, Mr. and Mrs. Henry D.
Ryder, Mr. and Mrs. Francis C.
Saunders, Dr. and Mrs. John W.

Saunders, Mrs. Lawrence
Sawyer, Mr. and Mrs. John E.
Schlesinger, Mrs. R. Walter
Scott, Mrs. George T.
Scott, Mrs. Norman E.
Sears, Mr. and Mrs. Harold B.
Segal, Dr. and Mrs. Sheldon J.
Shapiro, Mrs. Harriet S.
Shemin, Dr. and Mrs. David
Shepro, Dr. and Mrs. David
Smith, Mrs. Homer P.
Smith, Mr. VanDorn C.
Snider, Mr. Eliot
Specht, Mrs. Heinz
Spiegel, Dr. and Mrs. Melvin
Steele, Mrs. M. Evelyn
Steinbach, Mrs. H. B.
Stetten, Dr. and Mrs. DeWitt, Jr.
Stracher, Dr. and Mrs. Alfred
Stunkard, Dr. Horace
Sturtevant, Mrs. A. H.
Swanson, Dr. and Mrs. Carl P.
Swope, Mr. and Mrs. Gerard L.
Swope, Mrs. Gerard, Jr.
Taylor, Marjorie G.
Tietje, Mr. and Mrs. Emil D., Jr.
Todd, Mr. and Mrs. Gordon F.
Tolkan, Mr. and Mrs. Norman N.
Tompkins, Mrs. B. A.
Trager, Mrs. William
Troll, Dr. and Mrs. Walter
Tully, Mr. and Mrs. Gordon F.
Valois, Mr. and Mrs. John
Van Brunt, Mr. and Mrs. A. H., Jr.
Veeder, Mrs. Ronald A.
Waite, Mr. and Mrs. Charles E.
Waksman, Dr. and Mrs. Byron H.
Ware, Mr. and Mrs. J. Lindsay
Warren, Mrs. Shields
Watt, Mr. and Mrs. John B.
Weisberg, Mr. and Mrs. Alfred M.
Wheatley, Dr. Marjorie A.
Wheeler, Dr. and Mrs. Paul S.
Wheeler, Dr. and Mrs. Ralph E.
Whitney, Mr. and Mrs. Geoffrey G., Jr.
Wichterman, Dr. and Mrs. Ralph
Wickersham, Mr. and Mrs. A. A. Tilney
Wickersham, Mrs. James H., Jr.
Wilhelm, Dr. Hazel S.
Witmer, Dr. and Mrs. Enos E.
Wolfinsohn, Mr. and Mrs. Wolfe
Woodwell, Mrs. George
Yntema, Mrs. Chester L.
Zinn, Dr. and Mrs. Donald J.
Zipf, Dr. Elizabeth
Zwilling, Mrs. Edgar

## III. CERTIFICATE OF ORGANIZATION

(On File in the Office of the Secretary of the Commonwealth)

No. 3170

We, Alpheus Hyatt, President, William Stanford Stevens, Treasurer, and William T. Sedgwick, Edward G. Gardiner, Susan Mims and Charles Sedgwick Minot being a majority of the Trustees of the Marine Biological Laboratory in compliance with the requirements of the fourth section of chapter one hundred and fifteen of the Public Statutes do hereby certify that the following is a true copy of the agreement of association to constitute said Corporation, with the names of the subscribers thereto:-

We, whose names are hereto subscribed, do, by this agreement, associate ourselves with the intention to constitute a Corporation according to the provisions of the one hundred and fifteenth chapter of the Public Statutes of the Commonwealth of Massachusetts, and the Acts in amendment thereof and in addition thereto.

The name by which the Corporation shall be known is THE MARINE BIOLOGICAL LABORATORY.

The purpose for which the Corporation is constituted is to establish and maintain a laboratory or station for scientific study and investigations, and a school for instruction in biology and natural history.

The place within which the Corporation is established or located is the city of Boston within said Commonwealth.

The amount of its capital stock is none.

*In Witness Whereof*, we have hereunto set our hands, this twenty seventh day of February in the year eighteen hundred and eighty-eight, Alpheus Hyatt, Samuel Mills, William T. Sedgwick, Edward G. Gardiner, Charles Sedgwick Minot, William G. Farlow, William Stanford Stevens, Anna D. Phillips, Susan Mims, B. H. Van Vleck.

That the first meeting of the subscribers to said agreement was held on the thirteenth day of March in the year eighteen hundred and eighty-eight.

*In Witness Whereof*, we have hereunto signed our names, this thirteenth day of March in the year eighteen hundred and eighty-eight, Alpheus Hyatt, President, William Stanford Stevens, Treasurer, Edward G. Gardiner, William T. Sedgwick, Susan Mims, Charles Sedgwick Minot.

(Approved on March 20, 1888 as follows:

*I hereby certify* that it appears upon an examination of the within written certificate and the records of the corporation duly submitted to my inspection, that the requirements of sections one, two and three of chapter one hundred and fifteen, and sections eighteen, twenty and twenty-one of chapter one hundred and six, of the Public Statutes, have been complied with and I hereby approve said certificate this twentieth day of March A.D. eighteen hundred and eighty-eight.

CHARLES ENDICOTT
*Commissioner of Corporations*)

## IV. ARTICLES OF AMENDMENT

(On File in the Office of the Secretary of the Commonwealth)

We, James D. Ebert, President, and David Shepro, Clerk of the Marine Biological Laboratory, located at Woods Hole, Massachusetts 02543, do hereby certify that the following amendment to the Articles of Organization of the Corporation was duly adopted at a meeting held on August 15, 1975, as adjourned to August 29, 1975, by vote of 444 members, being at least two-thirds of its members legally qualified to vote in the meetings of the corporation:

VOTED: That the Certificate of Organization of this corporation be and it hereby is amended by the addition of the following provisions:

> "No Officer, Trustee or Corporate Member of the corporation shall be personally liable for the payment or satisfaction of any obligation or liabilities incurred as a result of, or otherwise in connection with, any commitments, agreements, activities or affairs of the corporation.
>
> "Except as otherwise specifically provided by the Bylaws of the corporation, meetings of the Corporate Members of the corporation may be held anywhere in the United States.

> "The Trustees of the corporation may make, amend or repeal the Bylaws of the corporation in whole or in part, except with respect to any provisions thereof which shall by law, this Certificate or the Bylaws of the corporation, require action by the Corporate Members."

The foregoing amendment will become effective when these articles of amendment are filed in accordance with Chapter 180, Section 7 of the General Laws unless these articles specify, in accordance with the vote adopting the amendment, a later effective date not more than thirty days after such filing, in which event the amendment will become effective on such later date.

In *Witness whereof and Under the Penalties of Perjury*, we have hereto signed our names this 2nd day of September, in the year 1975, James D. Ebert, President; David Shepro, Clerk.

(Approved on October 24, 1975, as follows:

I hereby approve the within articles of amendment and, the filing fee in the amount of $10 having been paid, said articles are deemed to have been filed with me this 24th day of October, 1975.
PAUL GUZZI
*Secretary of the Commonwealth*)

## V. BYLAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

(Revised August 11, 1978)

I. (A) The name of the Corporation shall be The Marine Biological Laboratory. The Corporation's purpose shall be to establish and maintain a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history.

(B) Marine Biological Laboratory admits students without regard to race, color, sex, national and ethnic origin to all the rights, privileges, programs and activities generally accorded or made available to students in its courses. It does not discriminate on the basis of race, color, sex, national and ethnic origin in employment, administration of its educational policies, admissions policies, scholarship and other programs.

II. (A) The members of the Corporation ("Members") shall consist of persons elected by the Board of Trustees, upon such terms and conditions and in accordance with such procedures, not inconsistent with law or these Bylaws, as may be determined by said Board of Trustees. Except as provided below, any Member may vote at any meeting, either in person or by proxy executed no more than six months prior to the date of such meeting. Members shall serve until their death or resignation unless earlier removed, with or without cause, by the affirmative vote of two-thirds of the Trustees then in office. Any member who has attained the age of seventy years or has retired from his home institution shall automatically be designated a Life Member provided he signifies his wish to retain his membership. Life Members shall not have the right to vote and shall not be assessed for dues.

(B) The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

III. The officers of the Corporation shall consist of a Chairman of the Board of Trustees, President, Director, Treasurer and Clerk, elected or appointed by the Trustees as set forth in Article IX.

IV. The Annual Meeting of the Members shall be held on the Friday following the Second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 a.m. Subject to the provisions of Article VIII(2), at such meeting the Members shall choose by ballot six Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the Members may be called by the Chairman or Trustees to be held at such time and place as may be designated.

V. Twenty five Members shall constitute a quorum at any meeting. Except as otherwise required by law or these Bylaws, the affirmative vote of a majority of the Members voting in person or by proxy at a meeting attended by a quorum (present in person or by proxy) shall constitute action on behalf of the Members.

VI. (A) Inasmuch as the time and place of the Annual Meeting of Members are fixed by these Bylaws, no notice of the Annual Meeting need be given. Notice of any special meeting of Members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least 15 days before such meeting, to each Member at his or her address as shown on the records of the Corporation.

(B) Any meeting of the Members may be adjourned to any other time and place by the vote of a majority of those Members present or represented at the meeting, whether or not such Members constitute a quorum. It shall not be necessary to notify any Member of any adjournment.

VII. The Annual Meeting of the Trustees shall be held promptly after the Annual Meeting of the Corporation at the Laboratory in Woods Hole, Massachusetts. Special meetings of the Trustees shall be called by the Chairman, the President, or by any seven Trustees, to be held at such time and place as may be designated. Notice of Trustees' meetings may be given orally, by telephone, telegraph or in writing; and notice given in time to enable the Trustees to attend, or in any case notice sent by mail or telegraph to a Trustee's usual or last known place or residence, at least one week before the meeting shall be sufficient. Notice of a meeting need not be given to any Trustee if a written waiver of notice, executed by him before or after the meeting is filed with the records of the meeting, or if he shall attend the meeting without protesting prior thereto or at its commencement the lack of notice to him.

VIII. (A) There shall be four groups of Trustees:

(1) Trustees (the "Corporate Trustees") elected by the Members according to such procedures, not inconsistent with these Bylaws, as the Trustees shall have determined. Except as provided below, such Trustees shall be divided into four classes of six, one class to be elected each year to serve for a term of four years. Such classes shall be designated by the year of expiration of their respective terms.

(2) Trustees ("Board Trustees") elected by the Trustees then in office according to such procedures, not inconsistent with these Bylaws, as the Trustees shall have determined. Except as provided below, such Board Trustees shall be divided into four classes of three, one class to be elected each year to serve for a term of four years. Such classes shall be designated by the year of expiration of their respective terms. It is contemplated that, unless otherwise determined by the Trustees for good reason, Board Trustees shall be individuals who have not been considered for election as Corporate Trustees.

(3) Trustees ex officio, who shall be the Chairman, the President, the Director, the Treasurer, and the Clerk.

(4) Trustees emeriti who shall include any Member who has attained the age of seventy years (or the age of sixty five and has retired from his home institution) and who has served a full elected term as a regular Trustee, provided he signifies his wish to serve the Laboratory in that capacity. Any Trustee who qualifies for emeritus status shall continue to serve as a

regular Trustee until the next Annual Meeting whereupon his office as regular Trustee shall become vacant and be filled by election by the Members or by the Board, as the case may be. The Trustees ex officio and emeriti shall have all the rights of the Trustees, except that Trustees emeriti shall not have the right to vote.

(B) The aggregate number of Corporate Trustees and Board Trustees elected in any year (excluding Trustees elected to fill vacancies which do not result from expiration of a term) shall not exceed nine. The number of Board Trustees so elected shall not exceed three and unless otherwise determined by vote of the Trustees, the number of Corporate Trustees so elected shall not exceed six.

(C) The Trustees and Officers shall hold their respective offices until their successors are chosen in their stead.

(D) Any Trustee may be removed from office at any time with or without cause, by vote of a majority of the Members entitled to vote in the election of Trustees; or for cause, by vote of two-thirds of the Trustees then in office. A Trustee may be removed for cause only if notice of such action shall have been given to all of the Trustees or Members entitled to vote, as the case may be, prior to the meeting at which such action is to be taken and if the Trustee so to be removed shall have been given reasonable notice and opportunity to be heard before the body proposing to remove him.

(E) Any vacancy in the number of Corporate Trustees, however arising, may be filled by the Trustees then in office unless and until filled by the Members at the next Annual Meeting. Any vacancy in the number of Board Trustees may be filled by the Trustees.

(F) A Corporate Trustee or a Board Trustee who has served an initial term of at least 2 years duration shall be eligible for re-election to a second term, but shall be ineligible for re-election to any subsequent term until two years have elapsed after he last served as Trustee.

IX. (A) The Trustees shall have the control and management of the affairs of the Corporation. They shall elect a Chairman of the Board of Trustees who shall be elected annually and shall serve until his successor is selected and qualified and who shall also preside at meetings of the Corporation. They shall elect a President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and Vice Chairman of meetings of the Corporation, and who shall be elected annually and shall serve until his successor is selected and qualified. They shall annually elect a Treasurer who shall serve until his successor is selected and qualified. They shall elect a Clerk (a resident of Massachusetts) who shall serve for a term of 4 years. Eligibility for re-election shall be in accordance with the content of Article VIII (F) as applied to Corporate or Board Trustees. They shall elect Board Trustees as described in Article VIII (B). They shall appoint a Director of the Laboratory for a term not to exceed five years, provided the term shall not exceed one year if the candidate has attained the age of 65 years prior to the date of the appointment. They may choose such other officers and agents as they may think best. They may fix the compensation and define the duties of all the officers and agents of the Corporation and may remove them at any time. They may fill vacancies occurring in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number as provided in Article X, and to delegate to such Committee such of their own powers as they may deem expedient in addition to those powers conferred by Article X. They shall from time to time elect Members to the Corporation upon such terms and conditions as they shall have determined, not inconsistent with law or these Bylaws.

(B) The Board of Trustees shall also have the power, by vote of a majority of the Trustees then in Office, to elect an Investment Committee and any other committee and, by like vote, to delegate thereto some or all of their powers except those which by law, the Articles of Organization or these Bylaws they are prohibited from delegating. The members of any such committee shall have such tenure and duties as the Trustees shall determine; provided that the Investment Committee, which shall oversee the management of the Corporation's endowment funds and marketable securities, shall include the Chairman of the Board of Trustees, the Treasurer of the Corporation, and the Chairman of the Corporation's Budget Committee, as *ex officio* members, together with such Trustees as may be required for not less than two-thirds of the Investment Committee to consist of Trustees. Except as otherwise provided by these Bylaws or determined by the Trustees, any such committee may make rules for the conduct of its business; but, unless otherwise provided by the Trustees or in such rules, its business shall be conducted as nearly as possible in the same manner as is provided by these Bylaws for the Trustees.

X. (A) The Executive Committee is hereby designated to consist of not more than ten members, including the ex officio Members (Chairman of the Board of Trustees, President, Director and Treasurer); and six additional Trustees, two of whom shall be elected by the Board of Trustees each year, to serve for a three-year term.

(B) The Chairman of the Board of Trustees shall act as Chairman of the Executive Committee, and the President as Vice Chairman. A majority of the members of the Executive Committee shall constitute a quorum and the affirmative vote of a majority of those voting at any meeting at which a quorum is present shall constitute action on behalf of the Executive Committee. The Executive Committee shall meet at such times and places and upon such notice and appoint such sub-committees as the Committee shall determine.

(C) The Executive Committee shall have and may exercise all the powers of the Board during the intervals between meetings of the Board of Trustees except those powers specifically withheld from time to time by vote of the Board or by law. The Executive Committee may also appoint such committees, including persons who are not Trustees, as it may from time to time approve to make recommendations with respect to matters to be acted upon by the Executive Committee or the Board of Trustees.

(D) The Executive Committee shall keep appropriate minutes of its meetings and its action shall be reported to the Board of Trustees.

(E) The elected Members of the Executive Committee shall constitute as a standing "Committee for the Nomination of Officers," responsible for making nominations, at each Annual Meeting of the Corporation, and of the Board of Trustees, for candidates to fill each office as the respective terms of office expire (Chairman of the Board, President, Director, Treasurer, and Clerk).

XI. A majority of the Trustees, the Executive Committee, or any other committee elected by the Trustees shall constitute a quorum; and a lesser number than a quorum may adjourn any meeting from time to time without further notice. At any meeting of the Trustees, the Executive Committee, or any other committee elected by the Trustees, the vote of a majority of those present, or such different vote as may be specified by law, the Articles of Organization or these Bylaws, shall be sufficient to take any action.

XII. Any action required or permitted to be taken at any meeting of the Trustees, the Executive Committee or any other committee elected by the Trustees as referred to under Article IX may be taken without a meeting if all of the Trustees or members of such committee, as the case may be, consent to the action in writing and such written consents are filed with the records of meetings. The Trustees or members of the Executive Committee or any other committee appointed by the Trustees may also participate in meeting by means of conference telephone, or otherwise take action in such a manner as may from time to time be permitted by law.

XIII. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees then in office.

XIV. These Bylaws may be amended by the affirmative vote of the Members at any meeting, provided that notice of the substance of the proposed amendment is stated in the notice of such meeting. As authorized by the Articles of Organization, the Trustees, by a majority of their number then in office, may also make, amend, or repeal these Bylaws, in whole or in part, except with respect to (a) the provisions of these Bylaws governing (i) the removal of Trustees and (ii) the amendment of these Bylaws and (b) any provisions of these Bylaws which by law, the Articles of Organization or these Bylaws, requires action by the Members.

No later than the time of giving notice of the meeting of Members next following the making, amending or repealing by the Trustees of any Bylaw, notice thereof stating the substance of such change shall be given to all Corporation Members entitled to vote on amending the Bylaws.

Any Bylaw adopted by the Trustees may be amended or repealed by the Members entitled to vote on amending the Bylaws.

XV. The account of the Treasurer shall be audited annually by a certified public accountant.

XVI. The Corporation will indemnify every person who is or was a trustee, officer or employee of the Corporation or a person who provides services without compensation to an Employee Benefit Plan maintained by the Corporation, for any liability (including reasonable costs of defense and settlement) arising by reason of any act or omission affecting an Employee Benefit Plan maintained by the Corporation or affecting the participants or beneficiaries of such Plan, including without limitation any damages, civil penalty or excise tax imposed pursuant to the Employee Retirement Income Security Act of 1974; provided, (1) that the Act or omission shall have occurred in the course of the person's service as trustee or officer of the Corporation or within the scope of the employment of an employee of the Corporation or in connection with a service provided without compensation to an Employee Benefit Plan maintained by the Corporation, (2) that the Act or omission be in good faith as determined by the Corporation (whose determination made in good faith and not arbitrarily or capriciously shall be conclusive), and (3) that the Corporation's obligation hereunder shall be offset to the extent of any otherwise applicable insurance coverage, under a policy maintained by the Corporation or any other person, or other source of indemnification.

## VI. REPORT OF THE DIRECTOR

### *Introduction*

The fashion of substituting for the traditional Christmas card a lengthy circular letter, often ill-duplicated and partially illegible, continues to gain devotees. These Director's Reports, like circular letters, are supposed to highlight events of the year past. I wish that they, too, had a deadline for composition close to the New Year. Then I should have before me several fresh examples, just arrived, of the circular Christmas letter. Some would be hortatory, but some would show me how *not* to proceed.

"Do not," they would warn, "try to cover everything that happened in the course of the year. Do not, as a slovenly alternative, deal only with what interests *you*, or solely with what has happened during the past two weeks. Do not assume that the recipients share your self-pity or self-aggrandizement. Do not select, from among all the subject possibilities, an undisciplined few."

What *not* to do is, unfortunately, easier here as elsewhere to exemplify than is the positive. If the life of a small family over the course of a year is unyielding to epistolary rules, then how intractable is a year's life of a great institution, if it must be encompassed within a report of a few pages that are less than instantly soporific! How much more intractable still is such a year's life when it has been marked by problems and progress, defeats and achievements, hopes proven vain and hopes fulfilled!

Still, like the correspondent who needed, last Christmas, to tell us the year's truth of her family in four pages (single-spaced) devoted to her husband's lower-back pain, I feel duty-bound to try. I, too am a neighbor to psychogenic discomforts. Having just now discovered her manuscript, mimeographed on green-tinted bond paper, amidst the schematics for our TV (which faltered at Christmas-time), I am newly mindful of the pitfalls of the *genre*, and of the laughter implicit in it. I have approached my task seriously enough this year, knowing that it will be judged dull or sharp according to the reader's preconceptions. But I have done the thing with a light heart, keeping before me a specific application of the great truth discovered by Will Rogers: "It's no trick being a humorist when you have the whole government working for you."

Think, then, of these few pages as a kind of MBL-family Christmas letter; arriving, not with the ice-crust that rims the Eel Pond in December, but with the mid-August rafts of cruising boats there. Think of it as an attempt to provide the absent member with some sense of what has happened since the last issue; but not with a comprehensive and representative summary of all the toothaches, the raffle-prizes, the IRS refund, the reports from teacher about our youngest's being cheeky again. There is a real comfort if the attempt fails: all the facts *are* summarized comprehensively in the remaining, and far more important, pages of this Annual Report. The financial ones carry the imprimatur, not only of our indispensable Treasurer, but also of our admirably scrupulous auditors.

### *Construction and Rehabilitation: Capital Campaign Phase I*

By the time this report is printed and distributed, all but a few minor components of the Phase I projects, planned and announced as the first steps of our Second Century Fund campaign, will have been funded and completed. The Environmental Sciences Center and the Candle House restoration were completed during 1981: Rehabilitation of Lillie began very early in 1982, that project having been fully subscribed during the prior year. Most of it will be done by June. The final jobs await a quieter time after Labor Day, when certain specialized equipment—such as the replacement for Lillie's wonderful elevator—will be delivered, and when there is once again the possibility that our contractor may park his large vehicles, trailer-trucks, and cranes behind Lillie without causing an outbreak of violence.

The Environmental Sciences Center has turned out far better than we dared to hope. The architects (Peirce, Pierce & Kramer) have done an imaginative job in an exceedingly difficult assignment: to convert an old, frame-and-shingle dormitory building to attractive office, conference, and teaching space, and then to attach the result, in an aesthetically acceptable way, to a modern, high-technology laboratory annex. All that has been done without compromising harmony of style and scale on the Quadrangle, and without ruining the view toward Great Harbor.

As good-looking as the facility is, it is also practical. All staff of the Ecosystems Center now occupy it, and it serves their purposes efficiently. There remains a significant amount of unfinished laboratory space that will provide for rational expansion and probably for transient use. The Environmental Sciences Center is a great step forward for MBL ecology. As is always the case in new laboratory buildings, minor problems have surfaced with heavy utilization of the facilities, but those are reparable and will soon be corrected.

The Candle House will surely be a model for similar restorations elsewhere. No important feature of its external appearance has been altered, but the interior is entirely new, and entirely satisfactory. Here, too the architects (Earl R. Flansburgh and Associates) combined sensitivity and good taste with technical skill, to bring a splendid old building, long dead so far as *habitability* was concerned, completely back to life. The administration are now housed there and have been since January, 1982. Unless my eyes and ears deceive me, they are housed in comfort and with decent furnishings consistent with the importance of their jobs. All of the space cleared in Lillie has become new laboratories or the expanded domain of the MBL Library.

Details of the Lillie rehabilitation, which is a project more costly and far more complex than the others of Phase I, are properly left to the Annual Report for 1982, since the bulk of the work will have been done in that year. 1981 was, nevertheless, the year that saw this undertaking, keystone of the entire plan for campus rehabilitation, funded. The Kresge Foundation's challenge grant ($0.5 million) was paid in recognition of our having raised another million (and somewhat more) in direct grants and pledges, including one of $100,000 from the MBL Associates in aid of the Auditorium renovations.

The donors to all these projects are too numerous to mention here. The gratitude owed them is too great to be expressed properly in a circular letter, but it is proper to note that all parts of the private sector were represented: Corporations, large and modest-sized; charitable Foundations; individuals, within and without the immediate MBL family, the gifts from those good people covering the entire range of possibilities, from bequests of real property to large, outright gifts of cash.

The work of Phase I will have cost, *in toto*, more than was planned in 1979, but by the standard of similar undertakings in other institutions, we have done remarkably well in these three inflation-plagued years. More importantly, the fund raising effort has accomplished more than simply to stay on schedule: it has in fact kept pace, overall, with the *actual* costs of the work. As will be evident in a later section of this Report, the MBL financial staff deserve special thanks for managing their part of this intricate undertaking.

As to the fund-raising itself, there is not much more to be said: it has kept pace. We could, and should, however, have done even better. The MBL's message is, as I know from experience, quite unique. That makes our case, once the chance to present it comes, differentiable from that of the host of schools, colleges, and social agencies now clamoring for the attention of private philanthropy. The clamor is, moreover, increasing steadily as government withdraws from the programs to which it has been committed for decades past.

Unique as the MBL's message is, therefore, and uniquely valuable for our culture as its services to biology are, there isn't much time to lose in fund raising: as the competition mounts, so will the negative effects of regional loyalties; arguments about "elitism;" the legitimate outcry for replacement of social services; and the likely continuation of economic troubles stemming from high energy costs and foreign industrial competition.

Progress toward the establishment of a competent, permanent in-house development capability was slow in 1981, for reasons beyond the possibility of control. All the work of funding Phase I had therefore to be done under the existing arrangements, with their heavy burdens upon the Director, his long-suffering secretary, and the Laboratory's external consultants. Effective as those arrangements may have been in terms of dollars won, and by comparison with the achievements of other institutions, they have not, in my opinion, been effective enough.

I am delighted to report, however, that in 1982 the MBL appointed a highly qualified Director of Development, with whose person and work many readers of this Report will have become familiar by the time of publication. We trust that there will be adequate time in which, with the aid of the new Development staff, we can achieve the stated goals of the Second Century Fund Campaign, and perhaps go beyond them, before the Laboratory's hundredth birthday.

### *Operations*

#### 1. *Financial*

Controller Edward Casey left the MBL in the early spring of 1981. The Associate Director, who remained in office for some eight months until, for personal and professional reasons, he found it necessary to resign, undertook as part of his assignment to stand in for the Controller. That effort had but limited success. The Associate Directorship—a position we have decided *not* to fill again—made too many other demands upon the incumbent's time. The search for a successor to Mr. Casey did not therefore begin seriously for a good many months.

Again, it gives me honest pleasure to report that a critical management problem *was* solved in due course: in April of 1982, Mr. John W. Speer, former chief financial officer of Rhode Island College, joined the Laboratory as its Controller. The high expectations of his performance we had, upon the basis of credentials and his important prior achievements, are being fulfilled. Objective evidence thereof is already to be seen everywhere in the Laboratory's financial activities.

It is, nevertheless, important to note that the Controller's department, every member of it, had to carry on for nearly a year *without* the authority and technical leadership of a Head. They responded as MBL staff seem nearly always to have done; quietly, and without complaint. The routine and the extra jobs were done: accounts payable, accounts receivable, payroll, personnel, the technically and psychologically demanding management of grants and contracts. They were done well.

I would like to think that these colleagues have understood the sincerity of my personal thanks, and I hope that somehow those of the entire MBL community will also be conveyed to them. I, for one, judge it no slight achievement for the Laboratory to have ended so complicated and management-deficient a year with its finances, for all practical purposes, in balance.

Among the threats and bad auguries of the past year in the domain of federal support for basic research, a positive event stands out for the MBL and the other U. S. A. marine laboratories. In the early spring of 1981, a group of Directors of those laboratories issued a brief report to the National Science Foundation. This document* resulted from earlier meetings, at first of the entire group, and later of a Steering Committee elected to prepare the draft. Its central concern was the financial plight of marine laboratories, especially those committed solely or largely to the study and research utilization of marine plants and animals.

Among its recommendations was the establishment within the N.S.F. of a special, inter-program funding mechanism, the purpose of which would be to provide "core" support for the maintenance and improvement of capital facilities at *these* laboratories. To the great credit of the Foundation's officers, they had provided partial financial support for the study, and among them are several who had anticipated its outcomes.

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* U. S. Marine Laboratories: A Plan for Modernization and Maintenance. J. D. Costlow, P. R. Gross, C. Pittendrigh, R. R. Strathmann, Steering Committee. Submitted to the National Science Foundation, dated February, 1981, under PCM-80-17003.

As it emerged, there was very little criticism of existing practices, but a good deal of argument for the need to rectify, by new practices, a potential threat to a group of indispensable national research facilities. After study of this report at all administrative levels, the Foundation responded by announcing a new program, quite close in design to what had been recommended. Initial-year funding is much less than the amount suggested, but more than we might have hoped for, considering the Foundation's already perilous budget situation for 1982.

What is important about these events, and about the evidence of good will and understanding within the N.S.F. administration, is not the detail of first-year funding, nor the announced rules (which are in fact reasonable). Rather, the new program formalizes recognition, by the key science agency of government, that marine biological laboratories are a *national* responsibility; they should, and probably will some day be, supported via more appropriate funding mechanisms. This is no more than has been done for many years in behalf of blue-water oceanography, but it is heartening to know that similar recognition of marine *biology* laboratories, of their missions and their accomplishments transcending descriptive marine biology, has emerged.

It would be difficult to overstate the potential importance of this for eventual rationalization of overhead cost recovery *systems* in institutions such as ours, quite independently of the quantitative arguments that rage, and will continue to rage, on the subject of overhead *costs*. For the MBL, where under-recovery of operating costs from the grants in whose behalf those costs are incurred has been a depressing fact of life, this is all good news.

### 2. *Library*

I shall not report here upon the Library's record of operations for the past year, since our Librarian has space of her own for the purpose elsewhere in this volume, but I cannot resist quoting from one of her recent memoranda: "At the present moment two electricians are working over my head installing new lighting, carpenters are drilling in the hall, and tile men are crawling on the future floor across the hall. So—being unable to do much of anything else I shall sit in the midst of chaos and send you thoughts. . . ."

Ms. Fessenden's written thoughts in this form can be pithy, often sufficiently pithier than the example to preclude their publishability in the learned press; but they always represent accurately the state of mind of the Library staff. In the present instance, that state of mind is influenced by Chaos, but—as I replied to her eventually—What a Lovely Chaos! After a very few months of it, we shall have that expanded and improved Library facility for which the MBL Corporation, and indeed the entire Woods Hole scientific community, have been agitating for decades.

### 3. *Buildings and Grounds*

Mr. Robert Gunning, who was eligible for a well-deserved and productive retirement in 1982, has been convinced, against his quite sensible initial decision, to remain on active duty as Head of the Department for another year. It was a generous decision under the circumstances; it means that we shall have the benefit of continuity in management and technical direction of that all-important component of the MBL staff during the rehabilitation of the Lillie Laboratory.

The negotiations alluded to had no negative effect upon the steady routine of the Department. In 1981, 45 laboratories in Lillie were renovated, including removal of the last of Dr. Drew's marvelously impregnable, obtrusive concrete sinks and tables, and their replacement with equipment made of stainless steel and fiberglass. All the old, cracked cast iron plumbing has been removed and replaced with fiberglass floor drains and polypropylene piping; sea water supply is now that long-sought dual system. First major steps in modernizing Lillie's heating systems were taken, including installation of thermostatic steam radiator valves and remote, wall-mounted thermostats in all laboratories.

For those many Corporation members and other investigators who are concerned about housing, it is pleasant to report that the B&G staff have refurbished completely the dormitory wing of the Brick Apartment House—an area that comprises three apartments, eighteen bedrooms, and three bathrooms. It will be ready for occupancy in the 1982 summer session.

### 4. *Marine Resources*

Having written a great many words of praise on this subject in last year's Director's Report, and since then in several other documents, I feel sure that more of them would be redundant here. Suffice it to say that having the new R/V GEMMA at its disposal has made the work of the department much easier than in the past. Not content with that relaxation of their difficulties, John Valois and his staff have responded by extending—without having been asked—its benefits to the entire MBL community, e.g., by adding another day to the squid delivery schedule. Satisfactory as GEMMA has proven, there remains an urgent need for properly operating and well-adapted vessels. While it was an earlier plan to eliminate from the fleet all wooden boats, the petrochemical origin of polyester resins has made the cost of new fiberglass boats prohibitive. Accordingly, the wooden R/V CIONA has had a complete refit, including much work on the hull and deck rig, with the result that this familiar workhorse, which has served the MBL for twenty years, should now have at least another ten in her.

Not mentioned in earlier reports is the work of the SCUBA diving team attached to this Department. It consists of three competent divers, whose equipment and work schedules are handled with far greater care than even the applicable regulations require. In consequence, such MBL regulars among marine animals as *Microciona*, *Spisula*, *Chaetopterus*, *Metridium*, and *Asterias* continue to be available for research. If these forms had to be dredged, rather than collected by divers, they would no longer be "available" in the sense of utility.

The new, but already productive collaboration between the Marine Resources Department and the Laboratory for Marine Animal Health deserves special mention, but more appropriately below, under the head of Research Programs.

### 5. *Public Information*

This Department was known until recently as "Public Relations," but the representations of an honored Trustee, more sensitive to language and titular affect (because of his profession, as well as in consequence of his good literary genes) than the rest of us, convinced us to change the name. Barbara Haskell was in charge through 1981 and until March, 1982, at which time she resigned because of the need to move away from the Cape. This will be a loss deeply felt, because under Haskell's direction and with her painstaking attention to style and content, all the MBL publications coming from the Department showed a discontinuously upward change in quality.

Those publications include more than the familiar MBL NEWSLETTER and NEXUS. Issued from the Department's office are such *ad hoc* literary objects (and they *have*, some of them, been literary) as news releases to the press and biographical sketches of speakers—as for the Friday Evening Lectures.

In 1981 the Department undertook a number of important, new, non-recurring responsibilities, such as collaboration with producers and other powerful persons of the television world, and with the editors and staff writers of commercial and corporate magazines. These collaborations yielded several important television events (*e.g.*, segments of a NOVA program), a splendid article in the Polaroid Corporation's CLOSE-UP, and references to MBL science and scientists in such national publications as NEWSWEEK.

Barbara Haskell's able and energetic assistant, Lee Anne Campbell, has agreed to serve, and is serving competently at the time of writing, as acting Public Information Officer, until such time after establishment of our new Development office as all concerned may consult together on possible changes in organization and size of the Department. It is a hopeful sign for the future that this, and a few other Departments at the MBL, have acquired the depth of skilled manpower to allow such flexibility.

### 6. *The George M. Gray Museum*

The principal function of our Gray Museum is (1) to assist MBL and outside investigators and students in the identification of local and regional species of plants and animals, and (2) to supply otherwise poorly accessible information on collection and maintenance of organisms. The museum contains several thousand preserved specimens of local animals, as well as sample forms inhabiting the waters from Maine to Virginia. The herbarium contains about 5,000 sheets, principally of Cape Cod and Islands species. Holdings of the museum have been extensively catalogued and checklisted: the Curators, of whom Dr. Wesley N. Tiffney is the Chief, are available to assist investigators Monday through Friday, for a total of 20 hours per week. Their guidance is easily arranged for, as is opening of the museum at special times, by appointment. This modest, but excellently operated activity serves several hundred users per year, for each of whom those services are very important indeed.

### 7. *Instructional Programs*

In 1981 the Laboratory offered seven regular summer courses, each of which was, by the test of critical external opinion (as mine is not: I think that they're the best biology courses in the world), up to the historic high standard of the species. The second offering of our newest course, the Biology of Parasitism, headed by John David, was even more exciting and better-received within the community—if such a thing is possible—than the first. Rudy Raff's direction of the Embryology course was efficient as before, and the course content remained a remarkable example of eclecticism surviving in the midst of scholarship and high technical standards. Ivan Valiela and John Teal co-directed the Marine Ecology course for the fifth year and agreed, upon request of the Director and the Committee on Instruction, to continue for a sixth while the course's problems (entirely in the category of financial support) and strengths (the existing syllabus; the extraordinary opportunities of surrounding landscapes; the absence of such courses in any of the area universities) are assessed carefully, and financial support for the successor is sought. There will be, I hasten to add, no problem in recruiting a new Instructor-in-Chief. It is our concern, however, to give him a proper start.

Harlyn Halvorson's second year as head of the new Microbial Ecology course was eminently successful, and his unusual skills (for a distinguished experimental scientist) in management and fund-seeking have benefited the students and the MBL in a multitude of ways. In the unique Neural Systems and Behavior course, Ronald Hoy was joined this year by Eduardo Macagno to form a co-directorate. For this course, as in all the others, we now have objective, external peer-group evidence to support our internal conviction (always dangerous when left to itself) of excellence.

Joel Rosenbaum succeeded K. VanHolde, for the 1981 Physiology offering, as Instructor-in-Chief. This old and distinguished course, which has one of the longest continuous records of training grant support, has now undergone one of its quinquennial changes of direction: neither abrupt nor in respect of intellectual rigor, but perceptible nevertheless. As might be expected, it now has a new commitment to the study of motility, cytoskeletal organization, and nucleo-cytoplasmic interactions at that level, and it is at the forefront of the field.

I might note here that being at the forefront does not preclude cyclicity: the last time this last emphasis was brought to the course it was done by Daniel Mazia, aeons ago, when the writer sat in on the lectures as an excuse for not starting to work on his thesis at the crack of dawn.

John Hildebrand and Tom Reese directed the Neurobiology Course for their second year, and that had two noteworthy outcomes. First, and by far the more important for the writer, who must pay attention to serious things, was their perfect adherence to budget, without any noticeable attrition of quality. Second was that, in connection with applications for continued funding, they sought some modest testimonials in the course's behalf from a number of the world's most eminent neuroscientists. The outcome, which should have been no surprise, since most of those are also alumni or associates of the course, was nevertheless gratifying: With no exception, those asked wrote letters to the course directors asserting—in sum—that the MBL summer Neurobiology course is the only one, and therefore the best, of its kind in the world; that it would be an unthinkable disservice to American neurobiology for its support to be diminished and its survival threatened.

Gerald Peters and Fred Ausubel paid us an extended visit during the summer of 1981 for purposes of planning the organization and funding of our next major offering in the plant sciences, a pilot version of which is to be mounted in 1982, and the definitive offering in 1983, funding and the goodwill of the vegetable gods permitting.

1981 versions of the January semester and the Short Courses were in the main as described in earlier reports. The details are given elsewhere in this volume. Both series have been a boon to the Laboratory and to the participants, as the records attest. There having been some important events and decisions for change in these programs in 1982, I leave the subject for much fuller discussion next year.

The Macy Scholars' Program and the Steps Toward Independence Program made their accustomed contributions in 1981. The comment applied to the January semester, above, in respect of 1982 changes, applies here as well.

Dr. Morton Maser, who is Assistant Director for Educational and Research Services, has been a dedicated impresario of those performances by which the needs of the instructional program, as disparate as Admissions and advertising, on the one hand, and service laboratories (*e.g.*, EM, hot lab), on the other, are met. Assisting him as Admissions Officer, Jane Leighton has maintained civility and decorum ("kept the lid on," as one of our *patois*-prone instructors defined it) in a busy office with heavy potential for disturbance of the peace. I refer, thereby, to the habit of *other* administrations with programs to which many want access and few can gain it; and for which funding and student support is a labyrinth, of *hiding* the Admissions office and officers.

The MBL does not hide its Admissions office. Yet it has been a quiet, efficient, and friendly place. This last leads me to render thanks also to Joan Howard, Grants and Contracts Officer and a member of the Controller's Department, whose threading the labyrinth of training and other grants, specifically in support of our courses, has been as skillful a performance as Jane Leighton's.

## *Research*

### 1. *Summer*

The MBL was full again in the summer of 1981, and the summer was again full in the other sense—of lectures, symposia, and demonstrations beyond the traditionally scheduled ones. There were, for example, not only the expected Friday Evening Lectures, but such special events as a lecture by Adrian Horridge, visiting from Australia, on the insect compound eye, and Lynn Margulis's Associates' Lecture entitled "The Earliest Life on Earth," making heavy and audience-enthralling use of the magic lantern. The Rockefeller Foundation and the MBL co-sponsored a week-long special lecture series on Scientific Information Systems and Information Retrieval, with such participants as Kenneth S. Warren, Eugene Garfield, William Goffman, and Frederick Mosteller. These sessions were well-attended, and, fitting to the new partnership of the MBL and the WHOI in library matters, were held alternately at the two institutions.

It would be asking for trouble to identify a specific subset of the summer's many research achievements as *particularly* noteworthy. I can quell disbelief of that assertion by analogy, in a domain with which all readers are surely familiar: the baroque Concerto Grosso.

Those who organize ensembles to play these wonderful works know that for acceptable results in public performance, the orchestra can tolerate no weak players. All must be at least highly competent; by preference, excellent. These concerti are scored for two groups of players (one small and one larger). One consists, usually, of the first-desk violinists (two), a violist, a cellist, perhaps cembalo. These are known as the *concertino*: they play the interesting melodic lines and sound the central harmonies. The second group provides back-up and is known, collectively, as *ripieni* (literally "fillers").

Suffice it to say that there is conflict: You cannot have a collection of first-class musicians agree among themselves about who is *ripieno* and who is *concertino*. Nor does drawing lots help. No sensible fiddler will risk on a coin-toss having to play endlessly repeated bass notes. He would rather fight. Yet you do need equally good musicians; and you do need *ripieni*.

I divulge, for the curious, the best of several solutions devised over the centuries since Torelli and Corelli, Vivaldi and Boccherini started all the trouble. It works for all but the most polished professional groups, where the need to earn a living transcends pride, and the players do as they are told in order to get paid.

The trick is never to emphasize the identities of lead and fill players. It is, if at all possible, to allow rotation from one part to another (within voices), but to contrive for your most trustworthy players to be in the first chairs on the night of a performance, having seen to it that they practiced the parts well. It is to avoid sedulously any congratulatory words about individual parts, but to dwell instead upon the marvels of the orchestra *as a whole.*

It should now be clear why I prefer to deal with the research accomplishments of summer investigators, in a full-house MBL summer, as though the whole population were a sort of *Virtuosi di Woods'ole.* And that would do little violence to the truth. They are. A glance at a partial record—the published abstracts of the General Scientific Meetings, in the October issue of the *Biological Bulletin*—will bear me out. And note, please, that it is a partial record only. Not all of the summer's results are communicated in that form. The eventual full-length papers that result from a summer's work at the MBL appear in a score of different journals, in several languages, and over the course of the next three or four years.

Any subset of those papers, collected by conscious effort, sustains the conclusion obtained from independent tests of quality, *e.g.*: research grant support in this competitive time; academic positions and honors; the eventual destinations and jobs of students and postdoctoral fellows who do research here with the principal investigators. It is that the summer research population at the MBL, like that of its course faculty, is drawn, not from the middle of a national achievement distribution, but very much from its high side.

I have been accused, once or twice, of "elitism" while presenting data in support of the above. If that is the definition of the word, then so be it. But I should point out that no rules of the MBL except peer-opinion require it to be so, and that the geographic, institutional, disciplinary, and socio-economic origins of the population in question are so extraordinarily broad as to make such a definition fatuous.

### 2. *Year Round Programs*

For purposes of reporting, the year round programs are much less of a problem. The year round research group at the MBL being of a size with some pretty large university Departments, it is possible at least to mention enough samples, if not all of the programs, to yield an impression of the spectrum of research interests and accomplishments. That, it turns out, is worth doing, for the spectrum, although not the size and the resulting interaction, is the same, summer and year round.

For as long as I can remember, the MBL has had a few distinguished *emeritii* and senior faculty of other institutions in residence and at work the year round. A splendid current example of the phenomenon is *D. Eugene Copeland*, Professor *emeritus* of Tulane University and Trustee *emeritus* of the MBL. Gene Copeland first retired and brought his productive research program to Woods Hole in 1977. Since then he has continued, with grant support from NIH and NSF, his important fine-structure studies on the teleost swim bladder and retina.

The work is of physiological significance, for the swim bladder wall can retain gases (such as $O_2$) against pressures as high as 300 atm, while there are mechanisms in the eye that raise the local oxygen tension twenty times higher than would be produced by the dissociation of oxyhemoglobin.

A part of Copeland's program requires work on the fish as soon as they are brought to the surface, and since deep-sea species are the ones of interest, this means work on large oceanographic vessels. Dr. Copeland has the interesting distinction of being the first MBL principal investigator to serve as a chief scientist aboard a WHOI research vessel.

The *NIH Laboratory of Biophysics*, William J. Adelman, Chief, is a large, year round *contract* program, *i.e.*, one in which the research is done, effectively, "on location" with respect to the parent organization, the National Institute of

Neurological and Communicative Diseases and Stroke. By that mechanism, the lead scientists are employees of the Institute, while the remainder of the program—space, facilities, staff (including scientists)—is provided and managed by the contractor, the MBL. The Laboratory of Biophysics, Woods Hole Unit, has two sections, one on neural membranes, Dr. Adelman in charge, and one on neural systems, Dr. Dan Alkon in charge. Both these programs are large enough to preclude even a summary of current activity, but activity there is in good supply.

The section on neural membranes is concerned with the structure and functions of neural cells at the ultrastructure and molecular levels. In it, advanced electron-optical, electrophysiological, computer, and mathematical methods are employed in the analysis of membrane ionic channels, models for their physical and electrical behavior, and the periodic structures of subcellular macromolecular arrays of the neuroplasm. Squid giant axons are the experimental material of most common use, but other marine animal preparations are employed as well.

The section on neural systems investigates the processing of information, especially in reference to learning, in simple neural networks and in the component cells of those networks. The preparation of primary interest in this group is the nudibranch mollusc, *Hermissenda crassicornis*, cultured in the laboratory. A broad range of electrophysiological, biochemical, morphological, developmental, and behavioral experiments is carried out on conditioned animals and their nervous systems.

Among the recent successes of this multilevel approach to a defined, whole-animal neural system has been the identification of complete sensory pathways responding to natural stimuli such as light and gravitational field. Changes in associative learning behavior can now be related specifically to altered properties of individual motor neurons.

This approach to the cellular analysis of learning, which is simultaneously integrative and reductionist, is receiving close attention from cognitive scientists, as well as from neurobiologists, across the country.

Dr. Shinya Inoué, who must here represent a considerably larger group of principal investigators in *cell and developmental biology* concerned with *cell motility and morphogenesis*, continues the development of his uniquely sophisticated video microscopy system. With its aid, he and Dr. L. G. Tilney have recently visualized directly, and analyzed the diffusion-limited kinetics of, actin polymerization at the tip of the growing acrosomal process (perforatorium).

Yuchiro Tanaka, one of the first two recipients of a Jean and Katsuma Dan Fellowship, came from Sugashima to work with Inoué for six months in 1981. Dr. Tanaka has discovered a reversible relaxation of the cleavage furrow in *Arbacia* eggs treated with Cytochalasin B or D. By a combination of time-lapse video microscopy and tracking of cortical pigment granules, local changes in structure of the cell cortex can be visualized, measured, and analyzed. These studies promise to contribute importantly to elucidation of the role of actin filaments in cytokinesis.

J. R. Whittaker, one of a group of MBL developmental biologists concerned with localization and chemical identification of *cytoplasmic morphogenetic determinants* in the early embryo, is also the Director of the *Boston University Marine Program* at the MBL, whose faculty are engaged in a broad range of other disciplines as well (*e.g.*, behavior, neurophysiology, cell biology, ecology), and in graduate education. Whittaker has recently succeeded in the remarkable feat of transferring cytoplasm from cells of the muscle lineage in ascidian embryos to those of the epidermal lineage, causing thereby the eventual expression of a characteristic muscle enzymatic activity (acetylcholinesterase) in progeny of the epidermal lineage, where the activity would not otherwise appear. This opens the way toward the long-sought test for the *chemical* identity of a specific morphogenetic determinant in a classically mosaic embryo.

Another large year round program, indeed, the largest now established at the MBL, is the *Ecosystems Center*, George M. Woodwell, Director. Its committed grant support in 1981 (some of it applicable, of course, to subsequent years) was more than $5 million. The scientific staff of the Center are a quintessentially *collaborative* group, each investigator lending his expertise to a range of Center projects. The senior staff of the Center includes a number of internationally recognized figures in ecology.

Biogeochemical cycles are among the major interests and their investigation entrains contributions from most of the staff. In this connection, they have recently refined their estimate of the global release of $CO_2$ to the atmosphere attributable to deforestation. It falls in the range of $2\text{–}5 \times 10^9$ metric tons annually, which figure is to be compared with an estimated release from combustion of fossil fuels, world-wide, at $5.2 \times 10^9$ metric tons. The implication of such figures, if both are nearly correct, for the origins and control of an ominously rising $CO_2$ load in the earth's gaseous envelope, will be obvious.

Another important contribution comes from the staff members investigating sulfur cycling. They have found that a major fraction of the net primary production of salt marshes flows through the sulfur cycle of water and sediments in the marsh. That the complex transformation of sulfur in salt marshes and in other parts of the coastal zone are energetically linked to photosynthesis is a significant finding for the important analysis of those transformations.

The *Laboratory of Sensory Physiology*, Edward F. MacNichol, Director, and Alan Fein, Deputy Director, accommodates the research of a group of some twelve resident investigators and up to seven visiting or collaborating scientists. The Laboratory centers its investigations on the physiology and biophysics of vision, particularly on the uniquely favorable experimental preparations available from marine animals. The study of cone pigments by microspectrophotometry of single receptors, a technology in which this laboratory has long been at the forefront, has recently been featured in major articles for the general reader interested in science (*Scientific American* and *The Sciences*).

Other work in progress and of great importance in visual physiology deals with the state of Ca, most of which seems *not* to be the free ion, in the receptors. Identification of the ligand and determination of its chemical structure is the goal of this work. A rapidly responding and reliable electrode for measurement of intracellular Ca in the ventral photoreceptors of *Limulus* has been developed, and is being employed for measurement of Ca during illumination and light adaptation. Results obtained thus far show that while the amount of intracellular calcium does change during excitation and in the course of adaptation, the Ca concentration is not a *direct* indicator of receptor sensitivity.

I mention now, to complete this survey of year round research, two examples of programs in *marine biomedicine*, the only area in which year round activity at the MBL has recently been allowed a significant net increase in size, space, and facilities.

*Dr. Carol Reinisch*, Associate Professor in the Tufts University School of Veterinary Medicine, is in residence at the MBL as a condition of her academic appointment and responsibilities. The appointment represents a step toward the establishment of closer, and eventually curricular, ties between that institution and the MBL. Carol Reinisch's research interests are in cellular immunology and in the pathology of marine invertebrate animals (which subject she and Mrs. Frederik Bang profess in the MBL's January Course under that name).

An interesting example of the confluence of her two research interests, and likewise of basic with applied research, is her current work on neoplasia in *Mya arenaria.* Aside from their inherent oncological interest, these studies, which have been in progress since October of 1981, are of practical toxicological and public health value: the animals in which tumor incidence is studied are collected (with cooperation of the Commonwealth of Massachusetts) from tidal flats closed to shellfishing because of pollution.

Hematopoietic tumors in these animals are not rare: the incidence is in the neighborhood of 15%. Dr. Reinisch has generated a series of monoclonal antibodies (in Balb/c By mice) to the neoplastic *Mya* cells. At least nine of these react with surface antigens of the tumor cells, but not with those of normal cells. The work has, therefore, two distinct, implicit future directions: (1) careful, large-scale epidemiological studies of tumor incidence, employing the sensitive new diagnostic tools, in "clean" as well as polluted environments (and using, also, *Mytilus* for a test of the generality of the *Mya* findings); and (2) identification, using electrophoresis, of the antigens being recognized by the monoclonal antibodies, and their subsequent isolation and purification.

The *Laboratory for Marine Animal Health*, whose director is *Prof. Louis Leibovitz*, of Cornell University, represents a new program that is year round offspring to the flourishing Aquatic and Veterinary Medicine Program ("Aquavet"), a collaboration among the MBL, Cornell, and the University of Pennsylvania; and an important step toward equipping the entire marine resources effort at the MBL, including the Department of that name, for the next decade.

Such a program of preparedness, for the one Department without which research at the MBL could not proceed for more than a few days, entails a measured response to, and a plan for dealing with, several threats: (1) instability, unpredictability, or even disappearance of populations of wild animals needed for researcch (*e.g.*, squid!); (2) unidentified, and hence uncontrollable disease within populations of specimens already collected and held; (3) failure of the very old wooden buildings, relics of simpler and easier times, in which the high-intensity and contemporaneous marine resources activities are housed. The list can go on.

The third of those threats has, as its response, our plans for a new Marine Resources Center. Other than to identify and create programs of research that will share such a facility with the Department and its staff, there is little more to do than to find the money. The first two, however, imply a newly urgent need for whole volumes of scientific information that does not yet exist, *e.g.*, (1) the practical ecology of forms needed now and in the future for research, and (2) a diagnostic pathology and clinical medicine of those animals.

The Aquavet program is concerned with establishment and codification—*i.e.*, with the *creation* of such a discipline. It is a young venture, but it has already proven successful. It is clear that the informed clinical approach, for which veterinarians are trained, can and does allow insight into the diseases of hitherto unstudied species, and that such insights suggest and imply practical methods of control.

Dr. Leibovitz, who is a distinguished pathologist, and whose research activities in that field continue as before, except that he is now in residence at the MBL year round, has also established a strong working relationship with John Valois, head of the Marine Resources Department, and with its staff. Not unexpectedly for those of us who know Valois, cooperation and interest have been mutual: the Department has collaborated fully with the new Laboratory for Marine Animal Health (LMAH) in mounting those of its programs that require access to Marine Resources facilities and procedures. There are already several such programs, and I can hope to accomplish no more here than to convey, by means of a list, an impression of their scope, their purposes, and their research components:

Morning Rounds, for health inspection of all animals maintained here, with a new system of records that document numbers of animals held, numbers removed, morbidity, and mortality each day.

Direct and immediate examination of sick and dead animals, followed by necropsy in a special area set aside for it, and, for cases of interest, by detailed diagnostic pathology.

Regular water quality testing.

After a year of operation, the LMAH emerges as a practical and valuable activity, certain to have its descendant(s) in a key role in the MBL's planned Marine Resources Center. Specific priorities for maintaining animal health have already appeared: temperature control, sediment control, prevention or minimization of abrasive injuries. A new *program* of preventive care is in the making. A large number of specific infections and contagious bacterial, viral, mycotic, and parasitic diseases have been diagnosed in species that are of concern to us.

As the experimental facilities improve, it will be possible to reproduce these diseases and to define the specific pathogenicities. Therewith, by the classical sequence of scientific medicine, will come control measures, and in some cases, cures. Such information is an indispensable adjunct to data on water quality limits, nutritional requirements, and specific environmental needs of each species. It should be very much easier to proceed from such a background to mariculture *proper*, *i.e.*, to raising animals of defined and appropriate genetic constitution, entirely in the laboratory, than to investigate culture systems by the "Try it and See What Happens" method.

There is no question that this can be done: it is already being done here for a few species, and more broadly applicable biological (*e.g.*, reproductive, developmental) and engineering data are accumulating rapidly. What is needed is to prepare deliberately for a time when we shall need, or want, to do the same for any other species that we must now hunt and capture in the wild.

### *H. B. Steinbach*

Over a long career in teaching undergraduates, graduate students, and postdoctoral fellows, I have come to appreciate the wisdom of G. B. Shaw's self-analysis: he claimed to have taken the greatest pains in deciding exactly what to say, and then to have said it with utmost levity. Although I do not claim any sort of kinship with GBS, it is the method I adopted for leading the reader into this rather lengthy and serious Report.

The method fails consistently, however, in one kind of communication: that having to do with the loss of a valued and beloved colleague. Even if the head were cunning enough, the heart does not permit it.

H. Burr Steinbach, former MBL Director, distinguished scientist, and uniquely successful administrator, died at his home in Woods Hole on December 21, 1981. All MBL regulars knew him well, for although he was a man who valued privacy, he was also very knowable. This was, I think, the result of a great and beneficial calm that lay at the core of his personality.

His mind was of a complexity consistent with the pioneering work he did in cellular physiology, and with his sensitivity to other complex people; yet he was a simple man, as easy for a child to talk to as for a committee of Nobel Laureates. He managed and manipulated some of the most troublesome organizations in existence (university Biology Departments are, of course, high on that list), and yet he managed, somehow, to convey to everyone a perfect openness.

For such a man, usually cheerful, able to do whatever he wanted to do, unused to any sort of physical complaint, it must have been especially terrible to discover himself in the grip of an incurable neurological disease, his most valued physical powers, such as that of speech, disappearing day by day. Yet even then his simplicity and decency, his openness, remained.

During the final months, when every few days he would take an increasingly difficult walk around Woods Hole, he stopped regularly at our office in Lillie, and conversed with us by use of a pencil and a writing-pad, hung on him like a necklace. Not a few jokes were exchanged. The other people in the office, not known for their readiness to drop what they are doing in order to visit, did so automatically and happily for Burr.

He died as he had lived: with dignity and humanity. His is a great loss that we must record among the events of 1981.

## VII. REPORT OF THE CONTROLLER

At the Executive Committee meeting of May 8, 1982 during which a considerable amount of time focused on financial matters, it was suggested that, as a part of the annual financial report, the Controller highlight the more significant factors that have dominated the financial situation during the past year. In response to that request, I am providing some information that members of the Corporation might find useful in evaluating our financial performance during 1981.

In unrestricted Current Funds, we had a fund balance reduction of $43,871 (exclusive of a $75,087 transfer to unrestricted Plant Funds). While the financial objective is a balanced budget, the small operating loss that was experienced is not considered significant in the larger view of overall financial operations. This is particularly so when one realizes that the value of MBL plant funds increased by over $1.5 million, a direct result of major renovation projects.

### *Revenues*

Overall, *unrestricted* revenues increased by almost 5 percent, which by contemporary economic standards must be considered at best "level funding." The two areas where reductions were experienced were in unrestricted gifts and investment income. Unrestricted gifts dropped by 20 percent, which might be a matter of serious concern if it were not for the fact that our restricted gifts, mostly from the Candle House and Lillie renovations, increased significantly over 1980. In total, gifts (unrestricted and restricted) amounted to over $1.8 million, which was slightly ahead of 1980.

Investment income was down 2 percent from 1980. This reflects a conscious decision by the MBL to shift a significant portion of its investments from income-producing to capital-growth stocks. While revenue was down 2 percent, the value of the investment portfolio increased by 5.7 percent, which is encouraging, given the overall performance of the stock market during 1981.

### *Expenditures*

Unrestricted expenditures increased by 17 percent, reflecting, for the most part, the continued and persistent problem of inflation. Increases in administration costs included efforts to improve our development and financial management programs. Instruction costs increased by 39 percent over 1980, which indicates new commitments to the educational programs. While "unrestricted" expenses for Library support were down 15 percent, this reduction was more than offset by an increase in "restricted" funding, resulting from more effective use of gifts in support of the Library. In fact, aggregate financial support for the Library increased by 17 percent. Costs of plant operations were up by 16 percent, a direct result of increases in heating and electricity charges.

As we move from 1981 to 1982, the financial report suggests several areas for immediate attention. First, efforts must be made to improve support via unrestricted gifts. Second, we must continue attempts to increase overhead yield. Third, a better and more timely system of budget and financial control management must be implemented. Finally, we must evaluate carefully the ways in which we are spending funds to ensure that expenditures are efficient, effective, and optimally controlled.

## VIII. REPORT OF THE TREASURER

The Laboratory struggled to hold operating expenses within its income in 1981. As the accompanying financial statements show, the effort was not *entirely* successful. Gross income increased minimally in comparison with 1980, but expenses rose significantly.

Mindful of inflation's toll, the Executive Committee has from time to time approved increases in laboratory space rents and various use charges and fees. Comparison of year to year operating income items shows the positive effects of these actions, as well as the caution with which they have been taken.

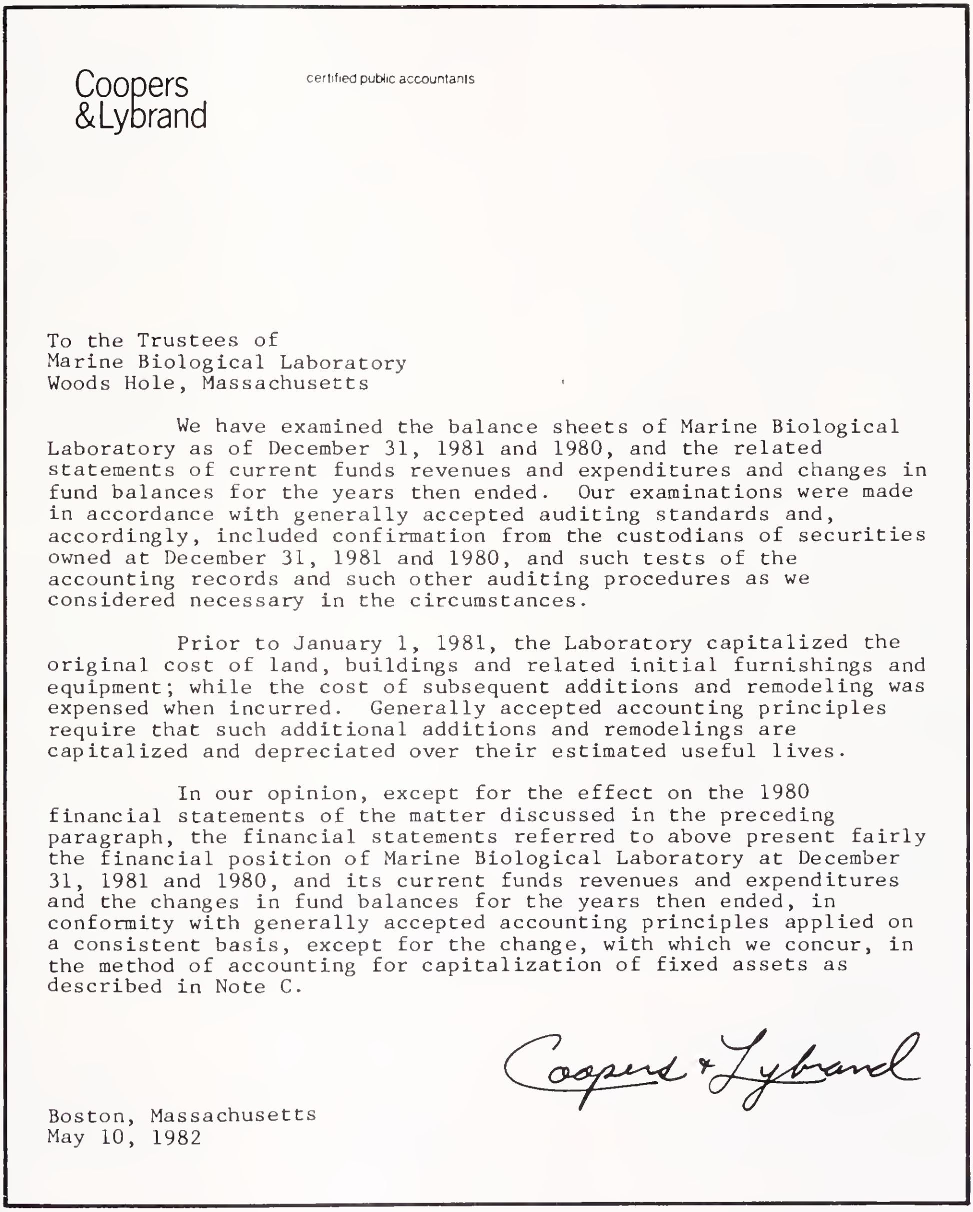
Investment income again contributed importantly to gross income. In part, this reflects the high interest rates which continued through 1981. Also significant, however, is the fact that the Laboratory's capital campaign generated funds which were invested profitably while awaiting application to their intended purposes within the campus rehabilitation and program plan (approved in 1979).

Inflation began to abate somewhat, late in 1981. Its effects therefore continued through most of the operating year to pose difficult management problems. Energy costs, for example, again exceeded expectations, causing plant operating expenses to escalate. Educational programs cost a good deal more to mount in 1981 than previously. Administrative expenses also increased. The expenses of fund raising, for example, were higher in 1981 as the capital campaign gathered momentum. Various steps to provide needed depth to the MBL's management capability also added to the year's administrative costs.

Stepped-up efforts to deal with the problem of delayed receipts were made in 1981. In most cases, the MBL's billed charges are paid by other institutions or by government agencies. User delays in approving and forwarding the Laboratory's invoices, coupled with the normal institutional delays in payment processing, create lags of many months to years in the receipt of payments. Fortunately, the Laboratory has not yet had to borrow in order to carry its receivables. Long payment delays nevertheless result in lost income, *e.g.*, interest, on monies owed to the MBL. Payment delays are especially unfair when the MBL finds itself, in effect, financing research expenses for which funds are available and reposing in other hands. For this reason, the Executive Committee has endorsed the policy of requiring advance payment for certain charges, beginning and enforceable in 1982.

The management and Executive Committee have given considerable attention to those trends and events which have the potential of altering significantly the Laboratory's future ability to fulfill its missions. Examples of such critical concerns are the effects of current and likely future cutbacks of government sponsorship for research and teaching; the greatly increased competition for private funds; and the growing sophistication of biological research technology, which requires ever-greater investment in facilities and equipment. Each such issue contains a major financial dimension.

Faced with the need to understand the detailed implications of such trends and events, the MBL is fortunate to have attracted to the position of Controller a person strongly qualified to direct the Laboratory's financial administration. Mr. John W. Speer, whose most recent service was as Controller and Chief Financial Officer of Rhode Island College, joined the management team of the MBL in April, 1982.



Coopers
&Lybrand
certified public accountants

To the Trustees of
Marine Biological Laboratory
Woods Hole, Massachusetts

We have examined the balance sheets of Marine Biological Laboratory as of December 31, 1981 and 1980, and the related statements of current funds revenues and expenditures and changes in fund balances for the years then ended. Our examinations were made in accordance with generally accepted auditing standards and, accordingly, included confirmation from the custodians of securities owned at December 31, 1981 and 1980, and such tests of the accounting records and such other auditing procedures as we considered necessary in the circumstances.

Prior to January 1, 1981, the Laboratory capitalized the original cost of land, buildings and related initial furnishings and equipment; while the cost of subsequent additions and remodeling was expensed when incurred. Generally accepted accounting principles require that such additional additions and remodelings are capitalized and depreciated over their estimated useful lives.

In our opinion, except for the effect on the 1980 financial statements of the matter discussed in the preceding paragraph, the financial statements referred to above present fairly the financial position of Marine Biological Laboratory at December 31, 1981 and 1980, and its current funds revenues and expenditures and the changes in fund balances for the years then ended, in conformity with generally accepted accounting principles applied on a consistent basis, except for the change, with which we concur, in the method of accounting for capitalization of fixed assets as described in Note C.

Coopers & Lybrand

Boston, Massachusetts
May 10, 1982

# MARINE BIOLOGICAL LABORATORY

## BALANCE SHEETS

### December 31, 1981 and 1980

| *Assets* | *1981* | *1980* |
|---|---|---|
| *Current funds:* | | |
| Unrestricted: | | |
| Cash and savings deposits | $ 212,262 | $ 363,907 |
| Money market securities | 1,850,000 | 1,250,000 |
| Accounts receivable, net of allowance for uncollectible accounts | 623,658 | 728,611 |
| Other assets | 19,531 | 5,004 |
| Due from (to) restricted current funds | (597,747) | 105,104 |
| Due to invested funds | (90,133) | (26,669) |
| Due to restricted plant fund | (720,535) | (1,052,224) |
| Total unrestricted | 1,297,036 | 1,373,733 |
| Restricted: | | |
| Accounts receivable | 346,828 | 733,431 |
| Investments, at cost (Notes B and F) | 2,179,531 | 2,085,227 |
| Due from (to) unrestricted current fund | 597,747 | (105,104) |
| Due from invested funds | 350,967 | 350,967 |
| Total restricted | 3,475,073 | 3,064,521 |
| Total current funds | $ 4,772,109 | $ 4,438,254 |
| *Invested funds:* | | |
| Investments, at cost (Notes B and F) | 4,488,885 | 4,219,999 |
| Due from unrestricted current fund | 90,133 | 26,669 |
| Due to restricted current funds | (350,967) | (350,967) |
| Total invested funds | $ 4,228,051 | $ 3,895,701 |
| *Plant funds:* | | |
| Unrestricted: | | |
| Land, buildings and equipment (Note C) | 14,907,184 | 12,940,384 |
| Less accumulated depreciation | 4,843,425 | 4,535,825 |
| Total unrestricted | 10,063,759 | 8,404,559 |
| Restricted: | | |
| Due from unrestricted current fund | 720,535 | 1,052,224 |
| Total restricted | 720,535 | 1,052,224 |
| Total plant funds | $10,784,294 | $ 9,456,783 |

The accompanying notes are an integral part of the financial statements.

# MARINE BIOLOGICAL LABORATORY

## Balance Sheets

### December 31, 1981 and 1980

| *Liabilities and Fund Balances* | *1981* | *1980* |
|---|---|---|
| *Current funds:* | | |
| Unrestricted: | | |
| Accounts payable and accrued expenses | $ 530,917 | $ 490,305 |
| Deferred income | 77,138 | 75,489 |
| Fund balance | 688,981 | 807,939 |
| Total unrestricted | 1,297,036 | 1,373,733 |
| Restricted funds: | | |
| Unexpended gifts and grants | 3,373,696 | 2,975,128 |
| Unexpended income of endowment funds | 101,377 | 89,393 |
| Total restricted | 3,475,073 | 3,064,521 |
| Total current funds | $ 4,772,109 | $4,438,254 |
| *Invested funds:* | | |
| Endowment funds | 2,218,669 | 2,077,500 |
| Quasi-endowment funds | 934,143 | 934,143 |
| Retirement fund (Note D) | 1,075,239 | 884,058 |
| Total invested funds | $ 4,228,051 | $3,895,701 |
| *Plant funds:* | | |
| Unrestricted | 10,063,759 | 8,404,559 |
| Restricted | 720,535 | 1,052,224 |
| Total plant funds | $10,784,294 | $9,456,783 |

The accompanying notes are an integral part of the financial statements.

## MARINE BIOLOGICAL LABORATORY

### Statements of Current Funds Revenues and Expenditures

for the years ended December 31, 1981 and 1980

| | *Unrestricted* | | *Restricted* | | *Total* | |
|---|---|---|---|---|---|---|
| | *1981* | *1980* | *1981* | *1980* | *1981* | *1980* |
| *Revenues:* | | | | | | |
| Instruction: | | | | | | |
| Tuition | $ 252,265 | $ 260,949 | $ 16,900 | $ 31,500 | $ 269,165 | $ 292,449 |
| Grants and contracts: | | | | | | |
| Government | 11,522 | 15,465 | 245,926 | 306,465 | 257,448 | 321,930 |
| Private | 28,124 | 22,000 | 113,958 | 192,224 | 142,082 | 214,224 |
| Research: | | | | | | |
| Laboratory rentals | 754,370 | 723,986 | | | 754,370 | 723,986 |
| Grants and contracts: | | | | | | |
| Government | 584,746 | 397,152 | 2,001,188 | 1,589,736 | 2,585,934 | 1,986,888 |
| Private | 43,477 | 104,530 | 220,109 | 134,709 | 263,586 | 239,239 |
| Dormitory | 381,954 | 368,600 | | | 381,954 | 368,600 |
| Dining Hall | 192,742 | 188,232 | | | 192,742 | 188,232 |
| Library | 144,154 | 133,728 | | | 144,154 | 133,728 |
| Biological Bulletin | 102,817 | 98,877 | | | 102,817 | 98,877 |
| Support departments: | | | | | | |
| Research services | 307,749 | 259,834 | | | 307,749 | 259,834 |
| Marine resources | 99,629 | 80,332 | | | 99,629 | 80,332 |
| Investment income | 287,081 | 292,722 | 283,574 | 289,672 | 570,655 | 582,394 |
| Gifts | 242,023 | 304,045 | 159,617 | 89,496 | 401,640 | 393,541 |
| Other | 118,865 | 142,828 | — | — | 118,865 | 142,828 |
| Total revenues | 3,551,518 | 3,393,280 | 3,041,272 | 2,633,802 | 6,592,790 | 6,027,082 |

| | Unrestricted | | Restricted | | Total | |
|---|---|---|---|---|---|---|
| *Expenditures:* | *1981* | *1980* | *1981* | *1980* | *1981* | *1980* |
| Instruction | 366,177 | 264,238 | 200,564 | 326,216 | 566,741 | 590,454 |
| Research | 3,488 | 1,726 | 2,420,567 | 1,945,869 | 2,424,055 | 1,947,595 |
| Scholarships and stipends | — | — | 265,565 | 295,937 | 265,565 | 295,937 |
| Dormitory | 184,034 | 165,688 | | | 184,034 | 165,688 |
| Dining Hall | 192,282 | 178,810 | | | 192,282 | 178,810 |
| Library | 231,530 | 272,261 | 146,029 | 49,784 | 377,559 | 322,045 |
| Biological Bulletin | 105,535 | 114,628 | | | 105,535 | 114,628 |
| Support departments: | | | | | | |
| Research services | 483,946 | 392,422 | | | 483,946 | 392,422 |
| Marine resources | 269,680 | 221,250 | | | 269,680 | 221,250 |
| Administration | 872,917 | 697,270 | 5,983 | 13,296 | 878,900 | 710,566 |
| Plant operation | 885,800 | 761,515 | 2,564 | 2,700 | 888,364 | 764,215 |
| Total expenditures | 3,595,389 | 3,069,808 | 3,041,272 | 2,633,802 | 6,636,661 | 5,703,610 |
| Excess (deficit) of revenues over expenditures | $ (43,871) | $ 323,472 | — | — | $ (43,871) | $ 323,472 |

The accompanying notes are an integral part of the financial statements.

# MARINE BIOLOGICAL LABORATORY

## STATEMENTS OF CHANGES IN FUND BALANCES

for the years ended December 31, 1981 and 1980

| | *Current Funds* | | *Invested Funds* | | | *Plant Funds* | | |
|---|---|---|---|---|---|---|---|---|
| | *Unrestricted* | *Restricted* | *Endowment* | *Quasi-Endowment* | *Retirement* | *Unrestricted* | *Restricted* | *Total All Funds* |
| Balances at December 31, 1979 | $ 720,614 | $2,692,819 | $1,918,170 | $934,143 | $ 746,597 | $ 8,007,053 | $ 300,000 | $15,319,396 |
| *Increases:* | | | | | | | | |
| Unrestricted current fund revenues | 2,796,513 | | | | | | | 2,796,513 |
| Grants | | 2,945,800 | | | | | | 2,945,800 |
| Gifts | 304,045 | 257,816 | | | | | 1,197,810 | 1,759,671 |
| Investment income | 292,722 | 309,535 | | | 44,904 | | | 647,161 |
| Realized net gains (losses) on sale of investments | | | 159,330 | | (671) | | | 158,659 |
| Addition to retirement fund | | | | | 117,557 | | | 117,557 |
| Tuition | | 31,500 | | | | | | 31,500 |
| *Decreases:* | | | | | | | | |
| Instruction, research and general expenditures | (3,069,808) | (2,633,802) | | | | | | (5,703,610) |
| Indirect costs | | (539,147) | | | | | | (539,147) |
| Payments to pensioners | | | | | (24,329) | | | (24,329) |
| Depreciation | | | | | | (284,227) | | (284,227) |
| Net change in fund balance before transfers | 323,472 | 371,702 | 159,330 | | 137,461 | (284,227) | 1,197,810 | 1,905,548 |
| *Transfers—additions (deductions):* | | | | | | | | |
| Additions to plant funds | (236,147) | | | | | 681,733 | (445,586) | — |
| Balances at December 31, 1980 | 807,939 | 3,064,521 | 2,077,500 | 934,143 | 884,058 | 8,404,559 | 1,052,224 | 17,224,944 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *Increases:* | | | | | | | | |
| Unrestricted current fund revenues | 3,022,414 | | | | | | | 3,022,414 |
| Grants | | 3,709,400 | | | | | | 3,709,400 |
| Gifts | 242,023 | 180,830 | 24,766 | | | | 1,365,000 | 1,812,619 |
| Investment income | 287,081 | 303,878 | | | 46,745 | | | 637,704 |
| Realized net gains on sale of investments | | 43,685 | 116,403 | | 33,096 | | | 193,184 |
| Realized net gains on disposal of fixed assets | | | | | | 62,950 | | 62,950 |
| Addition to retirement fund | | | | | 137,009 | | | 137,009 |
| Tuition | | 16,900 | | | | | | 16,900 |
| *Decreases:* | | | | | | | | |
| Instruction, research and general expenditures | (3,595,389) | (3,041,272) | | | | | | (6,636,661) |
| Indirect costs | | (667,869) | | | | | | (667,869) |
| Payment to pensioners | | | | | (25,669) | | | (25,669) |
| Depreciation | | | | | | (310,526) | | (310,526) |
| Net change in fund balance before transfers | (43,871) | 545,552 | 141,169 | | 191,181 | (247,576) | 1,365,000 | 1,951,455 |
| *Transfers—additions (deductions):* | | | | | | | | |
| Additions to plant funds | (75,087) | (135,000) | | | | 1,906,776 | (1,696,689) | — |
| Balances at December 31, 1981 | $ 688,981 | $3,475,073 | $2,218,669 | $934,143 | $1,075,239 | $10,063,759 | $ 720,535 | $19,176,399 |

The accompanying notes are an integral part of the financial statements.

# MARINE BIOLOGICAL LABORATORY

## NOTES TO FINANCIAL STATEMENTS

A. *Purpose of the Laboratory:*

The purpose of Marine Biological Laboratory (the "Laboratory") is to establish and maintain a laboratory or station for scientific study and investigations, and a school for instruction in biology and natural history.

B. *Significant Accounting Policies:*

*Basis of Presentation—Fund Accounting*

In order to ensure observance of limitations and restrictions placed on the use of resources available to the Laboratory, the accounts of the Laboratory are maintained in accordance with the principles of "fund accounting." This is the procedure by which resources are classified into separate funds in accordance with activities or objectives specified. In the accompanying financial statements, funds that have similar characteristics have been combined.

Externally restricted funds may only be utilized in accordance with the purposes established by the source of such funds. However, the Laboratory retains full control over the utilization of unrestricted funds. Restricted gifts, grants, and other restricted resources are accounted for in the appropriate restricted funds. Restricted current funds are reported as revenue when expended for current operating or other purposes. Unrestricted revenue is reported as revenue in the unrestricted current fund when earned.

Endowment funds are subject to restrictions requiring that the principal be invested with income available for use by the Laboratory. Quasi-endowment funds have been established by the Laboratory for the same purposes as endowment funds; however, any portion of these funds may be expended.

The financial statements for 1981 and 1980 reflect certain changes in format and presentation of the various funds. These changes have been made by the Laboratory to distinguish and identify the specific nature of certain restricted funds. Other reclassifications of amounts previously reported have been made to enhance the comparability of the financial statements.

*Investments*

Investments purchased by the Laboratory are carried at cost. Investments donated to the Laboratory are carried at fair market value at date received. For determination of gain or loss upon disposal of investments, cost is determined based on the average cost method.

*Investment Income and Distribution*

The Laboratory follows the accrual basis of accounting except that investment income is recorded on a cash basis. The difference between such basis and the accrual basis does not have a material effect on the determination of investment income earned on a year-to-year basis.

Investment income includes income from the investments of specific funds and from the pooled investment account. Income from the pooled investment account is distributed to the participating funds on the basis of the market value at the beginning of the quarter, adjusted for the cost of any additions or disposals during the quarter.

C. *Land, Buildings and Equipment:*

Following is a summary of the unrestricted plant fund assets:

| *Classification* | *1981* | *1980* |
|---|---|---|
| Land | $ 719,798 | $ 639,693 |
| Buildings | 12,535,197 | 10,694,543 |
| Equipment | 1,652,189 | 1,606,148 |
| | 14,907,184 | 12,940,384 |
| Less accumulated depreciation | 4,843,425 | 4,535,825 |
| | $10,063,759 | $ 8,404,559 |

The original cost of land, buildings and related initial furnishings is capitalized when assets are acquired. Prior to January 1, 1981 the cost of subsequent additions and remodeling was expensed when incurred which amounted to approximately $135,000 in 1980. Effective January 1, 1981 the Laboratory adopted the accounting policy of capitalizing such additions and remodeling in accordance with generally accepted accounting principles. For the year ended December 31, 1981 this change in accounting principle increased land, buildings and equipment by $794,000 and depreciation expense by $2,000. The financial statements have not been restated for the cumulative effect of this change since the amounts are not determinable.

Depreciation is computed using the straight-line method over estimated useful lives.

D. *Retirement Fund:*

The Laboratory has a noncontributory pension plan for substantially all full-time employees which complies with the requirements of the Employee Retirement Income Security Act of 1974. The actuarially determined pension expenses charged to operations in 1981 and 1980 were $137,009 and $117,557, respectively. The Laboratory's policy is to fund pension costs accrued, as determined under the aggregate level cost method. As of the latest valuation date, based on benefit information obtained January 1, 1982, the actuarial present values of vested and nonvested benefits, assuming an investment rate of return of 6%, were approximately $955,479 and $39,561, respectively. At January 1, 1982 net assets of the plan available for benefits, were approximately $1,055,861.

E. *Pledges and Grants:*

As of December 31, 1981 and 1980, the following amounts remain to be received on gifts and grants for specific research and instruction programs, and are expected to be received as follows:

| | *December 31, 1981* | | *December 31, 1980* | |
|---|---|---|---|---|
| | *Unrestricted* | *Restricted* | *Unrestricted* | *Restricted* |
| 1981 | | | $104,000 | $1,061,356 |
| 1982 | $20,000 | $ 96,800 | 50,000 | 66,333 |
| 1983 | | 95,000 | 5,000 | |
| 1984 | | 40,000 | | |
| | $20,000 | $231,800 | $159,000 | $1,127,689 |

In February 1979, the Laboratory initiated the MBL Second Century Fund, a phased effort, to secure $23 million in support of capital rehabilitation, new construction, and endowment. As of December 31, 1981, the Laboratory has received pledges related to this effort of approximately $4,000,000 of which a substantial portion has been collected.

F. *Investments:*

The following is a summary of the cost and market value of investment assets at December 31, 1981 and 1980, and the related investment income and disposition of investment income for the years ended December 31, 1981 and 1980.

| | *Cost* | | *Market* | | *Investment Income* | |
|---|---|---|---|---|---|---|
| | *1981* | *1980* | *1981* | *1980* | *1981* | *1980* |
| *Invested funds:* | | | | | | |
| U.S. Government securities | $ 918,742 | $ 910,077 | $ 831,790 | $ 814,012 | $102,201 | $ 88,349 |
| Corporate fixed income obligations | 217,920 | 803,462 | 167,930 | 669,561 | 28,024 | 91,199 |
| Common stocks | 3,116,473 | 2,414,311 | 3,504,027 | 3,137,223 | 154,454 | 133,246 |
| Preferred stocks | | | | | | 2,033 |
| Money market securities | 218,201 | 74,600 | 218,201 | 74,600 | 20,715 | 5,410 |
| Real estate (market at cost) | 17,549 | 17,549 | 17,549 | 17,549 | — | — |
| Total | $4,488,885 | $4,219,999 | $4,739,497 | $4,712,945 | 305,394 | 320,237 |
| Less custodian fees | | | | | 27,758 | 25,105 |
| | | | | | 277,636 | 295,132 |

| | *Cost* | | *Market* | | *Investment Income* | |
|---|---|---|---|---|---|---|
| *Current restricted funds:* | *1981* | *1980* | *1981* | *1980* | *1981* | *1980* |
| U.S. Government securities | 955,903 | 1,113,603 | 914,202 | 1,044,597 | 129,163 | 134,378 |
| Corporate fixed income obligations | 149,985 | 330,750 | 110,325 | 318,863 | 20,184 | 45,806 |
| Common stocks | 900,047 | 634,074 | 961,133 | 753,281 | 33,417 | 17,806 |
| Money market securities | 173,596 | 6,800 | 173,596 | 6,800 | 14,450 | 1,443 |
| | $2,179,531 | $2,085,227 | $2,159,256 | $2,123,541 | 197,214 | 199,433 |
| Less custodian fees | | | | | 9,126 | 5,992 |
| | | | | | 188,088 | 193,441 |
| *Current unrestricted funds:* | | | | | | |
| Earned on corporate savings accounts and money market funds | | | | | 171,980 | 158,588 |
| Investment income | | | | | $637,704 | $647,161 |
| *Disposition of investment income:* | | | | | | |
| Restricted for current use: | | | | | | |
| Utilized in current operations | | | | | 283,574 | 289,672 |
| Available for future operations | | | | | 20,304 | 19,863 |
| Total restricted current funds | | | | | 303,878 | 309,535 |
| Retirement fund | | | | | 46,745 | 44,904 |
| Unrestricted—utilized in current operations | | | | | 287,081 | 292,722 |
| | | | | | $637,704 | $647,161 |

## IX. REPORT OF THE LIBRARIAN

It was a year of planning and designing for the expansion of library space. The present collection is literally bursting off the shelves onto the side counters and tables, so the additional 5,000 square feet of space will be invaluable over the next ten years.

Discussions began in January with architects, a library consultant, users, and staff. The final plan will include the following:

(1) A tower will be constructed at the back of the Lillie Building to house a center staircase and elevator and provide access to all five stacks and three main floors.

(2) Wet labs on the third floor which are presently over library space will be eliminated. This area will become the future space for the entire "book" collection.

(3) The first floor administration area will house the archives and rare book collection, future microform machines and materials, and a conference room for Lillie scientists and library users.

Demolition and construction will begin in January, 1982, to be completed by the first of May in order to be ready for the summer users.

In 1981 we added 66 new journal titles to the collection, purchased 2,031 books, and filled over 4000 inter-library loan requests. We also added a 5600 Xerox machine to the copy service center, a Wang word processor, and an electronic typewriter for cataloging. Over 600 computer bibliographic searches were completed by three staff members who attended nine computer-update training sessions during the year.

## X. EDUCATIONAL PROGRAMS

### SUMMER

### Biology of Parasitism

*Instructor-in-chief*

David, John, Harvard Medical School

*Other faculty, staff, and lecturers*

Askenase, Philip, Yale University School of Medicine
Bang, Frederik, Johns Hopkins University
Carter, Richard, National Institutes of Health
Caulfield, John, Harvard Medical School
Cross, George, Wellcome Research Laboratories, United Kingdom
David, Peter, Harvard Medical School
David, Roberta, Harvard Medical School
Dessein, Alain, Harvard Medical School
Fearon, Douglas, Harvard Medical School
Gershon, Richard, Yale University School of Medicine
Gitler, Carlos, The Weizmann Institute of Science, Israel
Harn, Donald, Harvard Medical School
Hommel, Marcel, Harvard Medical School
Kafatos, Fotis, Harvard University
Karnovsky, Manfred, Harvard Medical School
Keusch, Gerald, Tufts University

Marsden, Philip, Universidade de Brasilia, Brazil
May, Robert, Princeton University
Miller, Louis, National Institutes of Health
Moser, Gina, Harvard Medical School
Nelson, George, Liverpool School of Tropical Medicine, United Kingdom
Nussenzweig, Ruth, New York University Medical Center
Pereira, Miercio, New England Medical Center
Pfefferkorn, Elmer, Dartmouth Medical School
Piessens, Willy, Harvard Medical School
Pratt, Diane, Harvard Medical School
Rifkin, Mary, Rockefeller University
Roberts, Bryan, Harvard Medical School
Sher, Alan, National Institutes of Health
Sherman, Irwin, University of California at Riverside
Simpson, Larry, University of California at Los Angeles
Spielman, Andrew, Harvard School of Public Health
Vincent, Albert, University of Southern Florida Medical Center
Warren, Kenneth, The Rockefeller Foundation
Wirth, Dyann, Harvard University

*Students*[1]

*Avila, Eva, Centro de Investigacion y Estudios Avanzados, Mexico
*Duerr, Ann, Massachusetts Institute of Technology
*Liberti, Pietro, Cell Biology Laboratory, Italy
*Lichtenstein, Lawrence, Johns Hopkins University
*Mendis, Kamini, University of Sri Lanka, Sri Lanka
*Ockenhouse, Christian, New York University Medical Center
*Perler, Francine, New England Biolabs, Inc.
*Philipp, Mario, National Institute for Medical Research, United Kingdom
*Safranek, Louis, Harvard University
*Santos, Isabel, Universidade Federal do Rio de Janeiro, Brazil
*Shapiro, Stuart, International Laboratory for Research on Animal Disease, Kenya
*Sidner, Richard, University of Cincinnati
*So, Maggie, Cold Spring Harbor Labs
*Unbekant, Lindsey, Harvard University
*Wahlgren, Carl, University of Stockholm, Sweden
*Winchell, Ellen, Johns Hopkins University

## EMBRYOLOGY

*Instructor-in-chief*

Raff, Rudolf, Indiana University

*Other faculty, staff, and lecturers*

Brandhorst, Bruce, McGill University, Canada
Bruskin, Arthur, Indiana University
Colot, Hildur, Brandeis University
Danilchik, Michael, University of Washington
Edgar, Robert, University of California at Santa Cruz
Guerrier, Pierre, Station Biologique, France
Hyman, Linda, Brandeis University
Jeffery, William, University of Texas

---

[1] All summer students listed completed the formal course programs. Asterisk indicates those completing post-course research sessions.

Klein, William, Indiana University
Kusch, Meredith, University of California at Santa Cruz
Moon, Randall, University of Washington
Moreau, Marc, Station Biologique, France
Neto, Rodrigo, Instituto de Biofisica, Brazil
Raff, Beth, Indiana University
Rankin, MaryAnn, University of Texas
Rosbash, Michael, Brandeis University
Ruderman, Joan, Harvard Medical School
Showman, Richard, Indiana University
Sowers, Louis, Indiana University
Tyner, Angelica, Indiana University
Vacquier, Vic, Scripps Institution of Oceanography

*Students*[1]

*Adelson, David, University of Hawaii
*Baltuch, Gordon, Harvard College
*Barnett, Faith, Harvard University
*Brown, Elizabeth, University of Washington
*Ferguson, James, Iowa State University
*Goralski, Thomas, Indiana University
*Groden, Joanna, Cornell Graduate School of Medical Sciences
*Henderson, Judith, State University of New York at Buffalo
*Hoefen, Paula, Pennsylvania State University
*Keenan, Katherine, Yeshiva College
*Krotoski, Danuta, Tulane University
*Lin, Peter, Johns Hopkins University
*McKinley, Dana, University of Miami
*Murtif, Vicki, Yale University
*Nicosia, Roberto, Medical College of Pennsylvania
*O'Brochta, David, University of California at Irvine
*Oliveira, Ana, Universidade Federal Do Rio De Janeiro, Brazil
*Shepard, Richard, University of Texas at Austin
*Sherman, Beth, State University of New York at Stony Brook
*Skuse, Gary, Syracuse University
*Spain, Lisa, Indiana University
*Tufaro, Frank, McGill University, Canada
*Wells, Dan, Indiana University

## Marine Ecology

*Instructors-in-chief*

Teal, John, Woods Hole Oceanographic Institution
Valiela, Ivan, Boston University

*Other faculty, staff, and lecturers*

Anderson, Don, Woods Hole Oceanographic Institution
Caraco, Nina, Boston University
Connell, Joseph, University of California at Santa Barbara
Dacey, John, Woods Hole Oceanographic Institution
Giblin, Anne, Boston University
Grassle, Frederick, Woods Hole Oceanographic Institution
Hargrave, Barry, Bedford Institute of Oceanography, Canada
Hobbie, John, Marine Biological Laboratory
Horgan, Erich, Marine Biological Laboratory

HUMES, ARTHUR, Boston University
JACKSON, J., Johns Hopkins University
JANNASCH, HOLGER, Woods Hole Oceanographic Institution
KOEHL, MIMI, University of California at Berkley
KORNBERG, HANS, Cambridge University, United Kingdom
LEVINTON, JEFF, State University of New York at Stony Brook
MADIN, LARRY, Woods Hole Oceanographic Institution
MANN, ROGER, Woods Hole Oceanographic Institution
NIXON, SCOTT, University of Rhode Island
ODUM, WILLIAM, University of Virginia
PETERSON, BRUCE, Marine Biological Laboratory
PETERSON, SUSAN, Woods Hole Oceanographic Institution
RIETSMA, CAROL, State University of New York at New Paltz
SEBENS, KENNETH, Harvard University
WATKINS, WILLIAM, Woods Hole Oceanographic Institution
WILTSE, WENDY, Williams College/Mystic Seaport
WITLACH, ROBERT, University of Connecticut

*Students*[1]

BAUER, JAMES, Boston University
CHOW, GEORGE, State University of New York at Buffalo
*DAVIS, JONATHAN, Yale University
*HALS, GARY, Capital University
*HUNTER, JUDY, Auburn University
*KEEN, SUSAN, University of Michigan
*LERER, DEBRA, University of Massachusetts
*LIGHT, JEFFREY, University of Colorado
*MARKS, BARBARA, Johns Hopkins University
*MERCURIO, KIMBERLEY, Ohio Wesleyan University
RADCLIFFE, GEORGE, Cathedral High School
*SHERRELL, ROBERT, Columbia University
STEPHAN, DIANNE, State University of New York at Syracuse
STOECKEL, MARK, Lafayette College
WAGNER, JEFFREY, State University of New York at Buffalo
*WAGNER, WENDY, Hanover College
*WALCH, MARIANNE, Harvard University
*WRIGHT, ANSON, Harvard University
WYNES, DAVID, Nichols College

## MICROBIAL ECOLOGY

*Instructor-in-chief*

HALVORSON, HARLYN, Brandeis University

*Other faculty, staff, and lecturers*

ALEXANDER, MARTIN, Cornell University
ATWOOD, KIMBEL, Columbia University
CITRI, NATHAN, Hebrew University, Israel
COHEN, YEHUDA, Hebrew University, Israel
DEMAIN, ARNOLD, Massachusetts Institute of Technology
DWORKIN, MARTIN, University of Minnesota
GIBSON, JANE, Cornell University
GRASSLE, FREDERICK, Woods Hole Oceanographic Institution
GREENBERG, E. P., Cornell University
HASTINGS, J. WOODLAND, Harvard University

Howe, Brian, Marine Biological Laboratory
Imhoff, Johannes, Institut Fur Mikrobiologie, Germany
Jannasch, Holger, Woods Hole Oceanographic Institution
Kaplan, Heidi, Cornell University
Keynan, Alex, Hebrew University, Israel
Klar, Amar. Cold Spring Harbor Labs
Kornberg, Hans, Cambridge University, United Kingdom
Leadbetter, Ed, University of Connecticut
MacLoeb, Robert, McGill University, Canada
Mahler, Henry, University of Texas
Margulis, Lynn, Boston University
Peck, Harry, University of Georgia
Poindexter, Jeanne, Public Health Research Institute at New York City
Reznikoff, William, University of Wisconsin
Romesser, James, Dupont Corporation
Rosenberg, Eugene, Tel Aviv University, Israel
Rownd, Robert, University of Wisconsin
Shapiro, James, University of Chicago
Shilo, Moishe, Hebrew University, Israel
Valiela, Ivan, Boston University
Vincent, Walter, University of Delaware

*Students*[1]

*Bingham, Peter, Harvard College
*Brill, Howard, Cornell University
*Castelfranco, Ann Marie, University of Iowa
*Cavanaugh, Colleen, Harvard University
*Durojaiye, Mustapha, Atlanta University
*Hall, Valerie, Nantucket High School
*Hamlett, Nancy, Towson State University
*Heuer, Ann, California State University
*Hong, Wek-Hee, Korea Advanced Institute of Science and Technology, Korea
*Jurick, Richard, University of Hawaii
*Kemp, Christopher, National Institutes of Health
*Kudlacz, Judy, University of Nebraska at Lincoln
*Miller, Molly, Massachusetts Institute of Technology/Woods Hole Oceanographic Institution
*Poonam, Gulati, Cornell University
*Roberts, Susan, University of California at San Diego
*Romero-Jarero, Jorge, Centro De Ciencias Del Mar Y Limnologia, Mexico
*Slock, James, Viterbo College
*Spencer, David, Indiana University
*Tomei, Francisco, Harvard University
*Van Rijn, Jaap, Hebrew University of Jerusalem, Israel

## Neural Systems and Behavior

*Instructors-in-chief*

Hoy, Ronald, Cornell University
Macagno, Eduardo, Columbia University

*Other faculty, staff, and lecturers*

Bennett, Michael, Albert Einstein College of Medicine
Calabrese, Ron, Harvard University
Carew, Thomas, Columbia University

DERIEMER, SUSAN, Yale University
DERBY, CHARLES, Boston University
EISNER, THOMAS, Cornell University
HARRIS-WARRICK, RON, Cornell University
HUBER, FRANZ, Max Planck Institute, Germany
KAHLE, GUNTHER, Free University of Berlin, Germany
KELLEY, DARCY, Princeton University
KRONENBERG, FREDI, New York, NY
LAYTON, J. KIERAN, Princeton University
LEVIN, MARGARET, Columbia University
LEVINTHAL, CYRUS, Columbia University
LEVINTHAL, FRANCOIS, Columbia University
MENZEL, RANDOLF, Free University of Berlin, Germany
MURPHY, ROD, State University of New York at Albany
NELSON, MARGARET, Cornell University
NOTTENBOHM, FERNANDO, Rockefeller University
PEARSON, KEIR, University of Alberta, Canada
THOMAS, JEFF, Columbia University
WALRATH, DANA ELIZABETH, Columbia University
WEEKS, JANICE, University of Washington
WOHLERS, DAVID, Cornell University

*Students*[1]

*BRODFUEHRER, PETER, University of Virginia
CARLIN, NORMAN, Harvard University
DON CARLOS, LYDIA, Northeastern Ohio Universities College of Medicine
DUMONT, JAMES, Stanford University
ELROD, SCOTT, Earlham College
*FALLS, DOUGLAS, State University of New York at Albany
GROSOF, DAVID, Harvard University
HALLANGER, ANN, University of Wisconsin at Madison
JUNG, LADONNA, Columbia University
KAULEN, PETER, Free University of Berlin, Germany
*KRIEGER, CHARLES, McGill University, Canada
*MOORE, DARRELL, University of Texas at Austin
NOVICKI, ANDREA, University of Hawaii
PALLAS, SARAH, Cornell University
POWELL, SUSAN, University of Oregon
QUINN, RICHARD, University of North Carolina at Charlotte
RAMIREZ, RAFAEL, Instituto Venezolano de Investigaciones Cientificas, Venezuela
RAYMOND, LYNN, Albert Einstein College of Medicine
ROBERTSON, GAIL, Washington University School of Medicine
*SHAMMA, SHIHAB, Stanford University
VALINSKI, WENDY, West Virginia University
YOUNG, ROBERT, Armed Forces Radiobiology Research Institute

## NEUROBIOLOGY

*Instructors-in-chief*

HILDEBRAND, JOHN, Columbia University
REESE, THOMAS, National Institutes of Health

*Other faculty, staff, and lecturers*

ALKON, DANIEL, National Institutes of Health/Marine Biological Laboratory
ALLEN, ROBERT, Dartmouth College

BARLOW, ROBERT, Syracuse University
BATTELLE, BARBARA, National Institutes of Health
BRANTON, DANIEL, Harvard University
BURD, GAIL, Massachusetts General Hospital
COULTER, JOSEPH, University of Texas
CRISTAKIS, NICHOLAS, Yale University
DOWLING, JOHN, Harvard University
DUNLAP, KATHLEEN, Tufts Medical School
FISCHBACH, GERALD, Harvard Medical School
FURSHPAN, EDWIN, Harvard Medical School
GELPERIN, ALAN, Princeton University
GOY, MICHAEL, Harvard Medical School
GRAHAM, WILLIAM, National Institutes of Health
HALBEISEN, JOHANNA
HALL, LINDA, Albert Einstein College of Medicine
HUTTNER, SUSANNE, University of California at Los Angeles
KANDEL, ERIC, Columbia University College of Physicians and Surgeons
KENT, KARLA, Columbia University
KRAVITZ, EDWARD, Harvard Medical School
LAFRATTA, JAMES, Harvard Medical School
LANDIS, DENNIS, Massachusetts General Hospital
LANDIS, STORY, Harvard Medical School
LASEK, RAY, Case Western Reserve University
LICHTMAN, JEFF, Harvard Medical School
MANSOUR, RANDA, University of North Carolina
MATSUMOTO, STEVEN, Harvard Medical School
MEINERTZHAGEN, IAN, Dalhousie University, Canada
MENZEL, RANDOLF, Free University of Berlin, Germany
NICHOLLS, JOHN, Stanford University
NISHI, RAE, Harvard Medical School
O'CONNELL, MAUREEN, National Institutes of Health
O'LAGUE, PAUL, University of California at Los Angeles
POTTER, DAVID, Harvard Medical School
RAHAMIMOFF, RAMI, Hebrew University Medical School, Israel
RAVIOLA, ELIO, Harvard Medical School
REESE, BONNIE, National Institutes of Health
ROBINSON, DAVID, Johns Hopkins University
SCHWAB, MARTIN, Max Planck Institute for Psychiatry, Germany
SCHWARTZ, JAMES, Columbia University College of Physicians and Surgeons
SHOTTON, DAVID, University of Oxford, United Kingdom
SMITH, STEVE, Yale University
VENKATESH, T. R., Albert Einstein College of Medicine
WALROND, JOHN, National Institutes of Health
WIESEL, TORSTEN, Harvard Medical School
WILLARD, MARK, Washington University
WYMAN, ROBERT, Yale University
ZIGMOND, RICHARD, Harvard Medical School
ZUKIN, SUZANNE, Albert Einstein College of Medicine

*Students*[1]

*BOYARSKY, GREGORY, Yale University
*CORMIER, SUSAN, Clark University
*FELS, GREGOR, Max Planck Institute, Germany
*HOSKINS, SALLY, University of Chicago
*HUBBARD, KAREN, Illinois Institute of Technology
*MATTOX, DOUGLAS, University of Texas Health Science Center

*Menco, Bernard, University of Utrecht, Netherlands
*Silberstein, Laura, University of California at San Francisco Medical School
*Stevens, Leslie, Harvard University
*Stuenkel, Edward, University of Hawaii
*Volman, Susan, Cornell University
*Wieland, Steven, Princeton University

## Physiology

*Instructor-in-chief*

Rosenbaum, Joel, Yale University

*Other faculty, staff, and lecturers*

Ackers, Gary, Johns Hopkins University
Albrecht, Guenter, Cold Spring Harbor Labs
Alexandraki, Despina, Harvard Medical School
Allen, Robert, Dartmouth College
Allewell, Norma, Wesleyan University
Altman, Sid, Yale University
Amos, Linda, Medical Research Council, United Kingdom
Ballinger, Dennis, Massachusetts Institute of Technology
Barnard, Steve, Connecticut College
Bennett, Vann, Johns Hopkins University
Chisholm, Rex, Massachusetts Institute of Technology
Chua, Nam Hi, Rockefeller University
Cleveland, Don, Johns Hopkins University
Cohen, Carl, St. Elizabeth's Hospital
Condeelis, John, Albert Einstein College of Medicine
Crain, William, The Worcester Foundation for Experimental Biology
Dentler, William, University of Kansas
DeTerra, Noel, Marine Biological Laboratory
Felsarfeld, Dan
Goldman, Ann, Northwestern University Medical School
Goldman, Robert, Carnegie-Mellon University
Gorovsky, Martin, University of Rochester
Greene, John, Massachusetts Institute of Technology
Guarante, Lenny, Harvard University
Hereford, Lynna, Brandeis University
Hobbie, Lawrence, Woods Hole, MA
Hunt, Timothy, Cambridge University, United Kingdom
Huxley, Hugh, Medical Research Council, United Kingdom
Inoué, Shinya, Marine Biological Laboratory
Jones, Jonathan, Northwestern University Medical School
Kaback, David, New Jersey Medical School
Kalfayan, Laura, Brandeis University
Kedes, Larry, Stanford University
Khan, Hamid, Northwestern University Medical School
Kilmartin, John, Medical Research Council, United Kingdom
Kamiya, Noburo, National Institute of Basic Biology, Japan
Lasek, Ray, Case Western Reserve University
Lodish, Havey, Massachusetts Institute of Technology
May, Grer, Yale University
Mayrand, Sandy, Worcester Foundation for Experimental Biology
McCarthy, Michael, Wesleyan University
McKintosh, Richard, University of Colorado

Mischke, Dietmar, Massachusetts Institute of Technology
Mitchell, David, Yale University
Mooseker, Mark, Yale University
Morris, Ron, Rutgers University
Murry, Andrew, Harvard University
Osley, Mary Ann, Brandeis University
Pardue, Mary Lou, Massachusetts Institute of Technology
Pederson, Thoru, Worcester Foundation for Experimental Biology
Pelham, Hugh, Carnegie Institute
Penman, Sheldon, Massachusetts Institute of Technology
Platt, Terry, Yale University
Pollard, Thomas, Johns Hopkins University
Reid, Martha, Earlham College
Ruderman, Joan, Harvard Medical School
Silfloe, Carolyn, Yale University
Sloboda, Roger, Dartmouth College
Steinburg, Eleanor, Oregon State University
Steinburg, Julie, Macalester College
Steinert, Peter, National Institutes of Health
Steitz, Joan, Yale University
Steitz, Thomas, Yale University
Stephens, Raymond, Marine Biological Laboratory
Talian, John, Northwestern University Medical School
Timasheff, Serge, Brandeis University
Vahey, Mary, Albert Einstein College of Medicine
Van Holde, Ken, University of Oregon
Wadsworth, Patricia, Dartmouth College
Weintraub, Harold, Cancer Research Institute
Wensink, Pieter, Brandeis University
Winkler, Matthew, University of California at Davis
Zachroff, Robert, Northwestern University Medical School

*Students*[1]

*Amos, William, University of Cambridge, United Kingdom
*Bandziulis, Raymond, Yale University
*Blank, Paul, Johns Hopkins University
Buhle, Emmett, Johns Hopkins School of Medicine
*Chambers, Carolyn, Xavier University
*Chou, Ying-Hao, University of Virginia
*DeSimone, Douglas, Dartmouth College
*Doniach, Tabitha, University of California at Santa Cruz
*Dunn-Coleman, Elaine, University of Virginia
*Green, Kathleen, Washington University
*Grimwade, Brian, Yale University
*Hager, Karl, Yale University
*Hering, Gordon, University of Wisconsin
Hildebrand, John, Stanford University
*Hollingsworth, Nancy, Oregon State University
*Hock, Rick, Albert Einstein College of Medicine
*Johnson, Nancy, Harvard University
*Karns, Larry, University of Virginia
[illegible] Yoshiteru, University of Tokyo, Japan
[illegible], Patricia, University of Pennsylvania
[illegible], University of Pennsylvania
[illegible], University of California at Berkeley
[illegible], St. Jude Children's Research Hospital

*MROCZKOWSKI, BARBARA, University of Connecticut
*RASMUSSEN, BETH, University of North Carolina
*ROBEY, ELLEN, University of Virginia
*SNABES, MICHAEL, Baylor College of Medicine
STULTS, LARRY, Johns Hopkins University
*UFKES, SUSAN, University of Massachusetts at Amherst
*WARD, GARY, University of California at San Diego

## JANUARY

### BASIC ECOLOGY AND MANAGEMENT OF RESOURCES

*Instructors-in-chief*

HOWARTH, ROBERT, Marine Biological Laboratory
WOODWELL, GEORGE, Marine Biological Laboratory

*Other faculty, staff, and lecturers*

BREWER, PETER, Woods Hole Oceanographic Institution
CAPUZZO, JUDITH, Woods Hole Oceanographic Institution
CHRISTENSEN, NORMAN, Duke University
COHEN, EDWARD, National Marine Fisheries Service
COMPTON, SARAH, U. S. Environmental Protection Agency
FOY, DOUGLAS, Conservation Law Foundation
GOLDBERG, EDWARD, Scripps Institution of Oceanography
GRASSLE, FREDERICK, Woods Hole Oceanographic Institution
GROSSLEIN, MARVIN, National Marine Fisheries Service
HEINSELMANN, MYRON, U. S. Forest Service
HINE, CAPT. LYNN, U. S. Coast Guard
HOBBIE, JOHN E., Marine Biological Laboratory
HOUGHTON, RICHARD A., Marine Biological Laboratory
MELILLO, JERRY M., Marine Biological Laboratory
MURPHY, EVELYN, Harvard University
PETERSON, BRUCE J., Marine Biological Laboratory
PISANO, VICTOR, TV Station 38, Boston
SANDERS, HOWARD, Woods Hole Oceanographic Institution
SHAVER, GAIUS R., Marine Biological Laboratory
SNEDECHER, JAMES, Anderson and Nichols
SPETH, GUS, Council on Environmental Quality
TEAL, JOHN, Woods Hole Oceanographic Institution
VACCARO, RALPH, Woods Hole Oceanographic Institution

*Students*

BROWNAWELL, BRUCE, Woods Hole Oceanographic Institution/Massachusetts Institute of Technology
DEMUTH, ROBERT, University of Alabama in Birmingham
ELBE, UTE, College of Santa Fe
GUSMAN, LINDA, Creve Coeur, MO
GUTJAHR, RUTH, National Marine Fisheries Service
HAAKONSEN, HARRY, Southern Connecticut State College
KOZAK, PATRICIA, University of New Hampshire
LADERMAN, RACHEL, Woods Hole, MA
LIPMAN, DEBORAH, Natural Resources Defense Council
LOWELL, VICTORIA, Tufts University
LOUGH, GAYLE, East Falmouth, MA
MOTT, RICHARD, Tulane University

Reese, Dwight, State University of New York at Stony Brook
Weems, Janice, Stetson University

## Behavior

*Instructor-in-chief*

Atema, Jelle, Boston University Marine Program/Marine Biological Laboratory

*Other faculty, staff, and lecturers*

Barlow, Robert, Syracuse University
Blough, Donald, Brown University
Bolling, Claude, Conservation de Paris, France
Bridges, Robert, Harvard Medical School
Brisbin, I. Lehr, Savannah River Ecology Program
Bryant, Bruce, Boston University Marine Program/Marine Biological Laboratory
Callard, Gloria, Harvard Medical School
Carey, Francis, Woods Hole Oceanographic Institution
Clark, Christopher, Rockefeller University
Derby, Charles, Boston University Marine Program/Marine Biological Laboratory
Dethier, Vincent, University of Massachusetts at Amherst
Edds, Peggy, University of Maryland
Elgin, Randall, Boston University Marine Program/Marine Biological Laboratory
Francis, Elizabeth, Bates College
Frazier, Jean, Brandeis University
Gerhardt, Carl, University of Missouri
Hain, James, University of Rhode Island
Hausfater, Glen, Cornell University
Ireland, Leonard, Bermuda Biological Station, Bermuda
Kalmijn, Adrianus, Woods Hole Oceanographic Institution
Kamil, Al, University of Massachusetts at Amherst
Kreithen, Mel, University of Pittsburgh
Langbauer, William, C. V. Whitney Laboratory
Levine, Joseph, Harvard University
Möller, Peter, American Museum of Natural History
Nyby, John, Lehigh University
Ristau, Carolyn, Rockefeller University
Stuart, Alastair, University of Massachusetts at Amherst
Sulzman, Frank, State University of New York at Binghamton
Swain, Tony, Boston University
Wilcox, Stimson, State University of New York at Binghamton
Williams, Timothy, Swarthmore College
Williams, Janet, Swarthmore College

*Students*

Baker, Tahirih, Jackson State University
Brown, Janice, Tougaloo College
Bryant, Donald, Boston University
Caraco, Nina, Boston University
Elliott, Wanda, Jackson State University
Ferme, Paola, Boston University
Fujita, Rodney, Boston University
Goldfarb, Shari, Cornell University
Moss, Anthony, Boston University
Schutrumpf, Andrew, Northeastern University
Segal, Yvette, Cornell University

## COMPARATIVE PATHOLOGY OF MARINE INVERTEBRATES

*Instructor-in-chief*

BANG, FREDERIK, Johns Hopkins University

*Other faculty, staff, and lecturers*

BANG, BETSY, Johns Hopkins University
CHANG, PEI WEN, University of Rhode Island
COOPER, KEITH, Thomas Jefferson University Medical School
DUCKLOW, HUGH, Columbia University
EDDS, KENNETH, State University of New York at Buffalo
ELSTON, RALPH, Cornell University
FARLEY, AUSTIN, National Marine Fisheries Service
LEVIN, JACK, Johns Hopkins University
MICHELSON, EDWARD, Harvard University
PEARCE, JACK, National Marine Fisheries Service
PRENDERGAST, ROBERT, Johns Hopkins University
REINISCH, CAROL, Harvard Medical School
STRANDBERG, JOHN, Johns Hopkins University
SCARBOROUGH, ANN, Johns Hopkins University

*Students*

ANDERSON, KENNETH, University of Chicago
FONTAINE, ANNE, Mount Holyoke College
GILLES, KAY, University of California at Santa Cruz
KLINGENSMITH, J. SCOTT, University of Mississippi Medical School
KOELLE, REINER, Eisenhower College
LANDY, RONALD, University of Pennsylvania
LEONARD, LESLIE, Johns Hopkins University
MANIGLIA, MARY ANN, Roosevelt University
MCCORMICK-RAY, M. GERALDINE, University of Virginia
ROCK, ALAN, University of Rhode Island
SHIFTLET, GEORGE, Erskine College
SILVERMAN, BARRY, Johns Hopkins University
SPEARS, CLIFTON, Dillard University

## DEVELOPMENTAL BIOLOGY

*Instructor-in-chief*

EDDS, KENNETH, State University of New York at Buffalo

*Other faculty, staff, and lecturers*

ALBERTINI, DAVID, Harvard Medical School
BEGG, DAVID, Harvard Medical School
BELL, EUGENE, Massachusetts Institute of Technology
COLEMAN, ANNETTE, Brown University
COLEMAN, JOHN, Brown University
FUJIWARA, KUIGI, Harvard Medical School
GERBI, SUSAN, Brown University
GOLD, BERT, Tufts University
GROSS, PAUL, Marine Biological Laboratory
HARRIGAN, JUNE, Marine Biological Laboratory
HEIPLE, JEANNE, Harvard University
HEPLER, PETER, University of Massachusetts at Amherst

INOUÉ, SHINYA, Marine Biological Laboratory
LINCK, RICHARD, Harvard Medical School
LUTZ, DOUGLAS, Marine Biological Laboratory
MARCUS, NANCY, Woods Hole Oceanographic Institution
MARIN, FRED, Brown University
MASER, MORTON, Marine Biological Laboratory
ROSENTHAL, ERIC, Harvard University
RUDERMAN, JOAN, Harvard Medical School
SCHUEL, HERBERT, State University of New York at Buffalo
TAMM, SIDNEY, Boston University/Marine Biological Laboratory
TAYLOR, D. LANSING, Harvard University
TROTT, THOMAS, Boston University
VOGEL, A. WAYNE, Harvard Medical School
YAMIN, MICHAEL, Marine Biological Laboratory

*Students*

ARCHIBALD, ASTON, Clark College
BERG, JOSEPH, California State College
COLEMAN, EDWARD, Texas Southern University
HAY, BRUCE, Claremont Men's College
JENKINS, GAYE, Dillard University
JOHNSON, DENISE, Texas Southern University
MENZEL, CHARLOTTE, Oberlin College
MOULDING, CHRISTOPHER, Harvard University
NEGUS, JAMES, Wofford College
PETTIS, RENEE, Texas Southern University
SANDERS, PAMELA, Texas Southern University
WALKER, ROSIE, Tougaloo College

## NEUROBIOLOGY

*Instructor-in-chief*

ALKON, DANIEL, National Institutes of Health/Marine Biological Laboratory

*Other faculty, staff, and lecturers*

ADELMAN, WILLIAM, National Institutes of Health/Marine Biological Laboratory
ATWOOD, HAROLD, University of Toronto
BARLOW, ROBERT, Syracuse University
BRIGHTMAN, MILTON, National Institutes of Health
CONNOR, JOHN, University of Illinois
DEFELICE, LOUIS, Emory University School of Medicine
DOWLING, JOHN, Harvard University
FARLEY, JOSEPH, Princeton University
FEIN, ALAN, Boston University Medical School/Marine Biological Laboratory
GOODMAN, STEVEN, Marine Biological Laboratory
GOVIND, C. K., University of Toronto, Canada
HAROSI, FERENCE, Boston University/Marine Biological Laboratory
JACKLET, JON, State University of New York at Albany
KAPLAN, EHUD, Rockefeller University
KRAVITZ, EDWARD, Harvard Medical School
KUZIRIAN, ALAN, National Institutes of Health/Marine Biological Laboratory
[illegible]RHENDLER, I. IZJA, National Institutes of Health/Marine Biological Laboratory
[illegible] SIMON, Harvard Medical School
[illegible], University of Massachusetts
[illegible] National Institutes of Health/Marine Biological Laboratory

PAPPAS, GEORGE, University of Illinois
PINTO, LAWRENCE, Purdue University
POTTER, DAVID, Harvard Medical School
PRICE, CHRISTOPHER, Boston University
RASMUSSEN, HOWARD, Yale University School of Medicine
RAYMOND, STEPHEN, Massachusetts Institute of Technology
RICHARDS, WILLIAM, Marine Biological Laboratory
SCHWARTZ, JAMES, Columbia University
SHEPHERD, GORDON, Yale University School of Medicine
SHOUKIMAS, JONATHAN, National Institutes of Health/Marine Biological Laboratory
SENFT, STEPHEN, University of Oregon
STEPHENS, RAYMOND, Boston University School of Medicine/Marine Biological Laboratory
SZUTS, ETE, Marine Biological Laboratory
TAMM, SIDNEY, Boston University/Marine Biological Laboratory
THOMPSON, CHARLES, University of Toronto, Canada
WALOGA, GERALDINE, Boston University School of Medicine
WEISS, THOMAS, Massachusetts Institute of Technology

*Students*

BOLDEN, MARSHA, Texas Southern University
BROOKS, CHARLES, Washington University
BRY, JOHN, Massachusetts General Hospital
CHING-JU, CHEN, University of Rhode Island
EISELE, LESLIE, University of Colorado at Boulder
FAMIGLIO, GREGORY, Massachusetts Institute of Technology
FREED, LAUREL, University of Bridgeport
GART, SERGE, Marlboro College
KRIKORIAN, JACQUELINE, University of Maryland School of Medicine
LANDRY, ANNE, Mount Holyoke College
LAYTON, BARRY, The Montreal General Hospital, Canada
LYDIC, RALPH, Harvard Medical School
NAFTOLOWITZ, DAVID, Amherst College
NORMAN, PHILLIPPA, Tougaloo College
PEARLSON, YALE, Tufts University
ROCHEL, SARAH, Roche Institute of Molecular Biology
SMITH, MARVA, Dillard University
ZEBLEY, ELMER, New College

## SHORT COURSES

### ANALYTICAL AND QUANTITATIVE LIGHT MICROSCOPY IN BIOLOGY, MEDICINE, AND MATERIALS SCIENCES

*Instructor-in-chief*

INOUÉ, SHINYA, Marine Biological Laboratory

*Other faculty, staff, and lecturers*

BELCHER, ARTHUR, Venus Scientific
CHIASSON, RICHARD, Olympus Corporation of America
COOMBS, GILLIAN, Marine Biological Laboratory
ELLIS, GORDON, University of Pennsylvania
ENDERS, REINHARD, E. Leitz, Inc.
HANAWAY, WINDHAM, Colorado Video
HAYES, THOMAS, University of North Carolina
HEIPLE, JEANNE, Harvard University

HINSCH, JAN, E. Leitz, Inc.
IIDA, HITOSHI, Hamamatsu Systems, Inc.
KELLER, ERNST, Carl Zeiss, Inc.
KLEIFGEN, JERRY, DAGE-MTI
LANGENBACH, UWE, Seiler Instruments (aus Jena)
LUTZ, DOUGLAS, Marine Biological Laboratory
OLWELL, PATRICIA, Seiler Instruments (aus Jena)
RIKUKAWA, KATSUJI, Nikon, Inc.
SALMON, EDWARD, University of North Carolina
SAWYER, WILLIAM, Carl Zeiss, Inc.
SCHEIER, KURT, Nikon, Inc.
SEIDLE, WALTER, Olympus Corporation of America
SUSSMAN, GARY, Crimson Camera Technical Sales, Inc.
TAYLOR, D. LANSING, Harvard University
TAYLOR, RICHARD, Colorado Video
THOMAS, PAUL, DAGE-MTI
WALLACE, PETER, Crimson Camera Technical Sales, Inc.

*Students*

ALDRICH, RICHARD, Yale University Medical School
BAJER, ANDREW, University of Oregon
BURGOS, MARIO, Harvard Medical School
CHAILLET, JOHN, Yale University Medical School
CHANDLER, WILLIAM, Yale University Medical School
COATES, THOMAS, Riley Hospital for Children
IRVING, MALCOLM, Yale University Medical School
KEITH, CHARLES, New York University Medical Center
MCMEEKIN, LINDA, American Cyanamid Company
MILLER, THOMAS, Harvard Medical School
ROMERO, FAUSTINO, Universidad Nacional Autonoma de Mexico, Mexico
SCHATTEN, GERALD, Florida State University
SOKOLOSKI, JOSEPH, University of Pennsylvania
WEISS, GILBERT, Life Savers, Inc.

## BIOLOGICAL ELECTRON MICROSCOPY FOR TECHNICIANS

*Instructor-in-chief*

MASER, MORTON, Marine Biological Laboratory

*Other faculty, staff, and lecturers*

ANTOL, JOE, Carl Zeiss, Inc.
COPELAND, D. EUGENE, Marine Biological Laboratory
GEISER, ALBERT, Hahnemann Medical Center
HOUGHTON, SUSAN, Woods Hole, MA
SAWYER, WILLIAM, Carl Zeiss, Inc.
UYDESS, IAN, Carl Zeiss, Inc.

*Students*

ARISSIAN, KOSTADINKA, Rockefeller University
BEETON, PHYLLIS, Temple University School of Medicine
BOCK, KITTY, Columbia University
BROWN, RUTH ANN, Washington State University
CAVANAUGH, COLLEEN, Marine Biological Laboratory
FERGUSON, PAULA, Maine Medical Center

GREEN, KAREN, Dillard University
KIMBERLY, PRISCILLA, University of Vermont
KOEHLER, INGRID, Brown University
LEMAY, PETER, College of the Holy Cross
SCHOUN, VERA, St. Vincent Charity Hospital
SIMS, DAVID, St. Vincent Charity Hospital

## ELECTRON MICROSCOPY IN THE BIOLOGICAL SCIENCES

*Instructors-in-chief*

BOWERS, BLAIR, National Institutes of Health
MASER, MORTON, Marine Biological Laboratory

*Other faculty, staff, and lecturers*

COOMBS, GILLIAN, Marine Biological Laboratory
COPELAND, D. EUGENE, Marine Biological Laboratory
HOHMAN, THOMAS, National Institutes of Health
HOUGHTON, SUSAN, Woods Hole, MA
PEACHEY, LEE, University of Pennsylvania
PORTER, KEITH, University of Colorado
WATERBURY, JOHN, Woods Hole Oceanographic Institution
WILLINGHAM, MARK, National Institutes of Health

*Students*

ANGELO, JEAN, Bowman Gray School of Medicine
BATTISTA, ARTHUR, New York University Medical School
BRYER, PAMELA, Bowdoin College
EELLS, THOMAS, Mary Imogene Bassett Hospital
FITE, KATHERINE, University of Massachusetts at Amherst
GALLANT, PAUL, National Institutes of Mental Health
HALL, MICHAEL, University of California School of Medicine at Los Angeles
HESSLER, ANITA, University of California at San Diego
JORDAN, THOMAS, Dillard University
MLADENOV, PHILIP, Mount Allison University, Canada
MURPHY-ULLRICH, JOANNE, University of Wisconsin
THOMAS, JUDITH, North Carolina State University

## FREEZE-ETCHING IN ELECTRON MICROSCOPY

*Instructor-in-chief*

STEERE, RUSSELL, U. S. Department of Agriculture

*Other faculty, staff, and lecturers*

COOMBS, GILLIAN, Marine Biological Laboratory
DEINAN, DERMOT, Polaron Instruments, Inc.
ERBE, ERIC, U. S. Department of Agriculture
GRAHAM, WILLIAM, National Institutes of Health
RASH, JOHN, Colorado State University

*Students*

BELL, BARBARA, Atlanta University
COCKERHAM, LORRIS, Armed Forces Radiobiology Research Institution
DUDEK, RONALD, East Carolina University Medical School
GEISER, ALBERT, Hahnemann Medical College and Hospital

KANNAN, MATHUR, McMaster Medical Centre, Canada
NUTTALL, ROBERT, Emory University
ROSLANSKY, PRISCILLA, Marine Biological Laboratory
RYERSE, JAN, St. Louis University School of Medicine
WATSON, GLEN, Florida State University

## MARICULTURE: CULTURE OF MARINE INVERTEBRATES FOR RESEARCH PURPOSES

*Instructor-in-chief*

BERG, CARL, Marine Biological Laboratory

*Other faculty, staff, and lecturers*

CAPUZZO, JUDITH, Woods Hole Oceanographic Institution
CAPO, THOMAS, Marine Biological Laboratory
DEAN, DAVID, University of Maine
DOYLE, ROGER, Dalhousie University, Canada
EARLY, GREGORY, New England Aquarium
ELSTON, RALPH, Cornell University
FUJITA, RODNEY, Marine Biological Laboratory
GARIBALDI, LOUIS, New England Aquarium
GOLDMAN, JOEL, Woods Hole Oceanographic Institution
GUILLARD, ROBERT, Woods Hole Oceanographic Institution
HARRIGAN, JUNE, Marine Biological Laboratory
HUGHES, JOHN, Massachusetts State Lobster Hatchery
HIXON, RAYMOND, Marine Biomedical Institute
MANN, ROGER, Woods Hole Oceanographic Institution
MARCUS, NANCY, Woods Hole Oceanographic Institution

*Students*

AGRAZ, FERNANDO, State Fisheries Laboratory, Mexico
ARCHER, VERNON, Jackson State University
CASTANARES, ERIC, Centro de Ciencias del Mar, Mexico
CARDELLI, LINDA, King James Shrimp Company
CHANG, ERNEST, Bodega Marine Laboratory
COLLINS, MARGERET, Howard University
DIOGUARDI, PAUL, Hackettstown, NJ
DURFEE, WAYNE, University of Rhode Island
GIBSON, BARBARA, University of Rhode Island
IGELSRUD, DON, The University of Calgary, Canada
MONTOYA, HAYDEE, University of Kansas
OLSON-MOORE, EDWARD, Sea World's Shark Institute
STICKLE, WILLIAM, Louisiana State University
ZIMMERMAN, JOHN, Yale School of Medicine

## OPTICAL MICROSCOPY AND IMAGING IN THE BIOMEDICAL SCIENCES

*Instructor-in-chief*

ALLEN, ROBERT, Dartmouth College

*Other faculty, staff, and lecturers*

ALLEN, NINA, Dartmouth College
BADY, MICHAEL, Nikon, Inc.
BROWN, DOUGLAS, Dartmouth College

Decker, Mel, Opti-Quip, Inc.
Gundlach, Heinz, Carl Zeiss, Inc., Germany
Hansen, Eric, Dartmouth College
Hayden, John, Dartmouth College
Horgan, Erich, Marine Biological Laboratory
Inoué, Shinya, Marine Biological Laboratory
Keller, Ernst, Carl Zeiss, Inc.
Kenealy, James, Hamamatsu Systems, Inc.
Maldari, Mario, Hamamatsu Systems, Inc.
Presley, Phil, Carl Zeiss, Inc.
Post, Nick, Nikon, Inc.
Phillips, Rick, Olympus Corporation of America
Scheier, Kurt, Nikon, Inc.
Scott, Eric, Venus Scientific
Scott, Martin, Eastman Kodak Company
Taylor, Marcia, Olympus Corporation of America
Vaughan, William, Vickers Instruments
Webb, Watt, Cornell University
Wick, Robert, Carl Zeiss, Inc.
Wong, Lenora, Dartmouth College

*Students*

Allred, Lawrence, S. C. Johnson & Son
Bowen, William, University of Arkansas at Little Rock
Brady, Scott, Case Western Reserve University
Cheney, Darwin, St. Elizabeth's Hospital
Cockerham, Lorris, Armed Forces Radiobiology Research Institute
Fisher, Richard, National Institutes of Health
Gilpin, R. R., Clarkson College of Technology
Kachar, Bechara, National Institutes of Health
Lindsey, James, University of California at San Diego School of Medicine
Palmer, Elizabeth, Instituto Politecnico Nacional, Mexico
Paolini, Paul, San Diego State University
Pesch, Gerald, U. S. Environmental Protection Agency
Rinnerthaler, Gottfried, Institute of Molecular Biology, Austria
Smith, Richard, University of Alberta, Canada
Simpson, Margaret, Sweet Briar College

## Protein Analysis by Polyacrylamide Gel Electrophoresis

*Instructor-in-chief*

Stephens, Raymond, Marine Biological Laboratory

*Other faculty, staff, and lecturers*

Herlands, Louis, The Rockefeller University
Horgan, Erich, Marine Biological Laboratory
Linck, Richard, Harvard Medical School
Piperno, Gianni, The Rockefeller University
Zweidler, Alfred, The Institute for Cancer Research

*Students*

Curfman, Gregory, Dartmouth-Hitchcock Medical Center
DiStefano, John, Northport Veterans Administration Medical Center
Dougherty, Edward, United States Department of Agriculture
Ferris, V. R., Purdue University

Floyd, Carl, Morehouse College
Floyd, Patricia, Morehouse College
Grebner, Eugene, Thomas Jefferson University
Hartzell, Charles, Alfred I. duPont Institute
Lotshaw, David, State University of New York at Albany
Ma, Nancy, Harvard Medical School
Severson, Arlen, University of Minnesota at Duluth School of Medicine
Shiffer, Kathleen, Milton S. Hershey Medical Center
Tassava, Roy, Ohio State University
Tucker, Robert, Johns Hopkins Oncology Center
Verrett, Joyce, Dillard University
Zimmer, Danna, Baylor College of Medicine

## QUANTITATIVE ANALYSIS OF ELECTRON MICROGRAPHS

*Instructor-in-chief*

Peachey, Lee, University of Pennsylvania

*Other faculty, staff, and lecturers*

Buschmann, Robert, Veterans Administration at Chicago
Haselgrove, John, University of Pennsylvania
Horgan, Erich, Marine Biological Laboratory
Palmer, Larry, University of Pennsylvania

*Students*

Benshalom, Gadi, National Institutes of Health
Botticelli, Lawrence, Stanford Medical School
Burnett, Paul, Polaroid Corporation
Contos, Nicholas, Florida State University
Fallon, John, Massachusetts General Hospital
Feser, Lee, Armed Forces Radiobiology Research Institute
Gregg, Marybelle, NCI—Frederick Cancer Research Facility
Hoftiezer, Virgil, Indiana University School of Medicine
Jones, Carl, University of Illinois Medical Center
Korte, Gary, Montefiore Hospital & Medical Center
Lee, Sheu-Ling, New England Medical Center Hospital
Lempka, Tim, Vanderbilt University Medical School
Nunzi, Maria, FIDIA Research Laboratories, Italy
Palatini, Dennis, American Cyanamid Company
Schotland, Donald, Hospital of the University of Pennsylvania
Wilson, Kathryn, Indiana University and Purdue University of Indianapolis

## SMALL COMPUTERS IN BIOMEDICAL RESEARCH

*Instructor-in-chief*

Palmer, Larry, University of Pennsylvania

*Other faculty, staff, and lecturers*

Blair, Richard, Dartmouth Medical School
Dezmelyk, Robert, Lab Computer Systems, Inc.
Horgan, Erich, Marine Biological Laboratory
Peachey, Lee, University of Pennsylvania

*Students*

Aigner, Thomas, University of Chicago
Aronson, Frank, Downstate Medical Center
Hoftiezer, Virgil, Indiana University School of Medicine
Kauer, John, Yale University
Kennedy, Brian, Yale University
Murphy, E. Hazel, Medical College of Pennsylvania
Murray, Marion, Medical College of Pennsylvania
Nunzi, Maria, FIDIA Research Laboratories, Italy
Obaid, Ana Lia, University of Pennsylvania
Oliver, Joseph, Medical College of Pennsylvania
Rhodes, Robert, Case Western Reserve University
Salzberg, Brian, University of Pennsylvania
Wilcox, R. Stimson, State University of New York at Binghamton
Wilkes, Mary, Medical College of Pennsylvania
Zingaro, Gloria, Medical College of Pennsylvania

# XI. RESEARCH AND TRAINING PROGRAMS

## SUMMER

### Principal Investigators

Adams, Paul R., University of Texas Medical Branch
Alberte, Randall S., University of Chicago
Allen, Robert D, Dartmouth College
Armstrong, Clay M., University of Pennsylvania
Arnold, John M., Kewalo Marine Laboratory
Barish, Michael E., University of California at Los Angeles
Barlow, Robert B., Syracuse University
Bartlett, Grant, Lab for Comparative Biochemistry
Beauge, Luis, Institution Investigacion Medica, Argentina
Begg, David A., Harvard Medical School
Bell, Wayne H., Hamilton College
Bennett, Michael V. L., Albert Einstein College of Medicine
Bezanilla, Francisco, University of California at Los Angeles
Bodznick, David, Wesleyan University
Borgese, Thomas A., CUNY, Lehman College
Boyer, Barbara C., Union College
Brenchley, Gayle A., University of California at Irvine
Brodwick, Malcolm S., University of Texas Medical Branch
Brown, Joel E., SUNY, Stony Brook
Brunken, William J., New York University Medical Center
Bullock, James O., Rush Presbyterian-Saint Luke's Medical Center
Burdick, Carolyn J., Brooklyn College
Burger, Max M., University of Basel, Switzerland
Cahalan, Michael D., University of California at Irvine
Cariello, Lucio, Stazione Zoologica di Napoli, Italy
Chad, John E., University of California at Los Angeles
Chang, Donald C., Baylor College of Medicine
Chappell, Richard L., CUNY, Hunter College
Charlton, Milton P., Ohio University
Cohen, Lawrence B., Yale University School of Medicine

Cohen, William D., CUNY, Hunter College
Cooperstein, Sherwin J., University of Connecticut Health Center
Dekin, Michael S., SUNY, Albany
Dentler, William L., University of Kansas
DeTerra, Noel, Hahnemann Medical College and Hospital
DeWeer, Paul J., Washington University School of Medicine
Dowling, John E., Harvard University
Dubois, Arthur, John B. Pierce Foundation Lab
Eaton, Douglas C., University of Texas Medical Branch
Eckert, Roger, University of California at Los Angeles
Farmanfarmaian, A., Rutgers University
Fernandez, Juilo, University of California at Los Angeles
Finger, Thomas E., University of Colorado
Fishman, Harvey M. University of Texas Medical Branch
French, Robert J., University of Maryland
Freschi, Joseph E., AFRRI
Frohlich, Amalie, Dalhousie University, Canada
Fussell, Catharine P., Pennsylvania State University
Gilbert, Daniel L., National Institutes of Health
Glanzman, David L., University of California at Los Angeles
Graf, Werner M., New York University Medical Center
Grosch, Daniel S., North Carolina State University
Guerrier, Pierre, Station Biologique, Roscoff, France
Haimo, Leah T., University of California at Riverside
Harding, Clifford V., Kresge Eye Institute
Haschemeyer, Audrey E. V., CUNY, Hunter College
Hernandez-Nicaise, Mari Luz, University Claude Bernard, France
Heuser, John, Washington University School of Medicine
Highstein, Stephen M., Albert Einstein College of Medicine
Hill, Susan D., Michigan State University
Hinch, Gertrude W., University of Southern Florida
Hoffmann, Richard J., Iowa State University
Hogan, James C., Yale University
Hoskin, Francis C. G., Illinois Institute of Technology
Humphreys, Tom, Kewalo Marine Laboratory
Hunt, R. Kevin, Johns Hopkins University
Ilan, Joseph, Case Western Reserve University
Ingoglia, Nicholas A., New Jersey Medical School
Ireland, Leonard, Bermuda Biological Station, England
James-Kracke, Marilyn, Washington University School of Medicine
Josephson, R. K., University of California at Irvine
Joyner, Ronald W., University of Iowa
Kaminer, Benjamin, Boston University School of Medicine
Kamiya, Noburo, National Institute for Basic Biology, Okazaki, Japan
Kao, C. Y., SUNY, Downstate Medical Center
Kilduff, Thomas, Stanford University
Kirsch, Glenn E., Rutgers University
Koehl, Mimi A. R., University of California at Berkeley
Kusano, Kiyoshi, Illinois Institution of Technology
Landowne, David, University of Miami
Lasek, Raymond, Case Western Reserve University
Laufer, Hans, University of Connecticut
Lee, John J., CUNY, City College
Levin, Jack, Johns Hopkins Hospital
Lipicky, Raymond J., National Institutes of Health
Llano, Isabel, University of California at Los Angeles

Llinas. R., New York University Medical Center
Loewenstein, Werner R., University of Miami School of Medicine
Longo, Frank J., University of Iowa
Lyttle, C. Richard, University of Pennsylvania
Maglott, Donna R., Howard University
Matsumura, Fumio, Michigan State University
Mauzerall, David, Rockefeller University
McKinney, Leslie C., Washington University
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## XII. HONORS

### FRIDAY EVENING LECTURES

GERHARDT, CARL, University of Missouri at Columbia, January 9, "*Sound Pattern Recognition in North American Tree Frogs: Neurobiological Implications*"

RASMUSSEN, HOWARD, Yale University, January 16, "*Calcium and Cyclic-AMP as Synarchic Messengers*"

LIEM, KAREL, Harvard University, January 23, "*Functional Morphology of the Feeding Apparatus of Fishes: Do Fish Defy Gauss' Principle?*"

DARNELL, JAMES E. Rockefeller University, June 26, "*Consideration of Animal Cell Function and Evolution*"

HOBBIE, JOHN E., Marine Biological Laboratory, July 3, "*Process Regulation in an Arctic Ecosystem*"

HEUSER, JOHN, Washington University School of Medicine, July 9, 10, Forbes Lectures. I. "*Structural Basis of Synaptic Transmission*" II. "*A 3-D Journey Through the Interior of Nerve and Muscle Cells*"

NICHOLLS, JOHN G., Stanford University, July 17, Lang Lecture, "*One Cell at a Time: The Analysis of a Simple Nervous System*"

STEITZ, JOAN A., Yale University, July 24, "*Autoantibodies as Probes for Small Ribonucleoproteins from Eukaryotes*"

MCINTOSH, J. RICHARD, University of Colorado, Boulder, July 31, "*Mitotic Mechanism: Ever Interesting, Still Elusive*"

SAGER, RUTH, Sidney Farber Cancer Institute, August 7, "*DNA Methylation: From* Chlamydomonas *to Cancer*"

INOUÉ, SHINYA, Marine Biological Laboratory, August 14, "*Form, Movement, and Life: Adventures in Light Microscopy*"

GROSS, JEROME, Massachusetts General Hospital, August 21, Zwilling Lecture, "*Regulation of Collagenase by Cell-Cell Interactions*"

SOMERO, GEORGE N., Scripps Institution of Oceanography, August 28, "*Protein Adaptation to the Physical Environment: Discerning Basic Molecular 'Themes' Through the Study of Their 'Variations'* "

### CHARLES A. LINDBERGH LECTURES IN ECOLOGY

MANN, KENNETH H., Bedford Institute of Oceanography, June 24, "*Management of Resources in the Coastal Zone:* Laminaria *and Lobsters in Nova Scotia*"

LOVEJOY, THOMAS E., World Wildlife Fund, U. S., July 8, "*Conserving Wildlife in a Fragmented World*"
BOLIN, BERT, University of Stockholm, July 29, "*Man's Interference with the Biosphere on a Global Scale*"

## ASSOCIATES' LECTURE

MARGULIS, LYNN, Boston University, August 1, "*The Earliest Life on Earth*"

## SPECIAL LECTURE

HORRIDGE, G. ADRIAN, Australian National University, July 12, "*New Work on the Insect Compound Eye*"

## ROCKEFELLER FOUNDATION LECTURE SERIES "UNDERSTANDING SCIENTIFIC INFORMATION SYSTEMS AND OPTIMIZING INFORMATION RETRIEVAL"

GOFFMAN, WILLIAM, Case Western Reserve University, July 13, "*The Ecology of the Biomedical Literature*"
WARREN, KENNETH S., Rockefeller Foundation, July 14, "*The Quantitative and Qualitative Structures of the Biomedical Literature*"
MOSTELLER, FREDERICK, Harvard School of Public Health, July 15, "*Design and Evaluation of Biomedical Studies*"
GOFFMAN, WILLIAM, Case Western Reserve University, July 16, "*Information Retrieval Strategies*"
GARFIELD, EUGENE, Institute for Scientific Information, July 17, "*Information Retrieval Systems*"

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"*Coupling between Horizontal Cells in the Carp Retina Examined by Diffusion of Lucifer Yellow.*"

By AKIMICHI KANEKO, National Institute for Physiological Sciences, Japan, and ANN E. STUART, University of North Carolina

"*An Optical Determination of the Resistance in Series with the Axolemma of* Loligo pealei."

By BRIAN M. SALZBERG, University of Pennsylvania, FRANCISCO BEZANILLA, University of California, Los Angeles, and H. V. DAVILA, Universidad Los Andes, Venezuela

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Walker, Allen R.
Whittaker, William A.
Wuethrich, Lisa
Wyttenbach, Robert A.

Reference: *Biol. Bull.* **163:** 108–130. (August, 1982)

# FISH GILL IONIC TRANSPORT: METHODS AND MODELS

DAVID H. EVANS[1], J. B. CLAIBORNE[2,†], LINDA FARMER[2], CHARLES MALLERY[2], AND EDWARD J. KRASNY, JR.[3]

[1]*Department of Zoology, University of Florida, Gainesville, FL 32611;* [2]*Department of Biology, University of Miami, Coral Gables, FL 33124; and* [3]*Department of Physiology and Biophysics, University of Alabama, Birmingham, AL 35294*

## INTRODUCTION

Because fishes, like all aquatic vertebrates except the marine hagfishes, maintain the $Na^+$ and $Cl^-$ content of their body fluids distinctly different from either their freshwater or marine environment, they face a constant net movement of salts and water* across their permeable membrane (predominantly the branchial epithelium). Thus, freshwater fishes (which are hyperosmotic to the medium) presumably face a net loss of NaCl and net influx of water. The reverse is presumably true for the hypo-osmotic marine fishes, *i.e.* they face a net gain of NaCl and loss of water. The general mechanisms which fishes utilize to balance these net salt and water movements were first outlined by Homer Smith (1930) and have been more recently reviewed rather extensively (Potts and Parry, 1964; Maetz, 1974; Kirschner, 1977, 1979; Evans, 1979, 1980a). In the past few years it has become increasingly obvious that rather complex, but quite intriguing, mechanisms of ion transport are resident in the epithelium of at least the teleost fish gill** (Maetz and Bornancin, 1975; Maetz *et al.*, 1976; Kirschner, 1977, 1979, 1980; Potts, 1977; Evans, 1979, 1980b, 1982a).

It is not the aim of the present review to carefully re-examine the data which have been discussed in these reviews. Instead, we propose to examine some recent techniques which have been employed to attempt to more carefully delineate the mechanisms of fish branchial ionic transport. In the process we intend to describe what, and what not, these techniques can tell us about this system as well as what we think we know about the various ionic transport mechanisms, and where we think we should be heading in the future.

Since many of the techniques have been developed in the past 20 years to avoid some of the problems of *in vivo* studies, it is appropriate to begin with a description of whole animal kinetic and electrochemical studies.

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Abbreviations: IPHP, isolated, perfused head preparation; TAP, triaminopyrimidine; TEP, transepithelial potential.

* Since the elasmobranch fishes (sharks, skates, *etc.*) maintain isotonicity to sea water *via* the retention of urea, they are an exception to this statement about net movements of water. However, it is important to note that their body fluids contain significantly less NaCl than sea water, so they still face net influxes of these ions in sea water (see Evans, 1979, for a more complete examination of elasmobranch osmoregulation).

** Henceforth we will be dealing with teleost fish, with only slight reference to the elasmobranchs, until the last section of this review.

## The Intact Animal

Investigations of possible mechanisms of gill transport actually started when Krogh (1939) demonstrated that the head end of goldfish (*Carassius auratus*) was able to extract $Na^+$ and $Cl^-$ from solutions independently of each other. He proposed that, since the uptake mechanisms were parallel but uncoupled, there were probably ionic exchange systems involved to maintain some semblance of electroneutrality. He suggested that the $Na^+$ uptake might be coupled to $NH_4^+$ extrusion and that $Cl^-$ uptake might be coupled to $HCO_3^-$ efflux. In an early study utilizing radioisotopes Maetz and Garcia Romeu (1964) supported Krogh's proposition by demonstrating that $Na^+$ and $Cl^-$ uptake could be stimulated, independently of each other, by injecting, respectively, $NH_4^+$ or $HCO_3^-$ into the blood of the goldfish. Addition of the same substances to the external medium inhibited $Na^+$ and $Cl^-$ uptake, respectively. Since the uptakes could be measured with $^{22}Na$ and $^{36}Cl$, this study represented a significant step forward from earlier studies which relied on chemical analysis to monitor net fluxes. Nevertheless it also demonstrates at least two pitfalls of the majority of whole-animal studies, even to the present time. $Na^+$ and $Cl^-$ are ions and their movements are therefore affected by electrical potentials as well as chemical gradients. Therefore it is possible that an experimental manipulation (in this case ionic substitution) which results in an alteration of the movement of an ion produces this change, not through a direct effect on some sort of ionic exchange system, but by altering the electrical gradient (transepithelial potential, TEP) across the epithelium in question. In other words, in the Maetz and Garcia Romeu (1964) experiments, it is possible (for example) that injection of $(NH_4)_2SO_4$ altered some TEP across the fish, made the blood more electronegative (relative to the fresh water) and thereby stimulated a passive uptake of $Na^+$. Corresponding arguments could be applied to the effects of $KHCO_3$ injections on $Cl^-$ uptake and the effects of external additions of either substance on $Na^+$ and $Cl^-$ uptake. Thus, firm statements about chemical *vs* electrical couplings simply cannot be made without concomitant measurements of the TEP.

The other pitfall of whole-animal studies (as demonstrated by the early work of Maetz and Garcia Romeu, 1964) is that specific alteration of the composition of the blood of intact animals is nearly impossible. For example the injection of $(NH_4)_2SO_4$ into the blood could have lowered the pH of the blood and thereby stimulated a $Na^+/H^+$ rather than $Na^+/NH_4^+$ exchange as proposed by the authors. Kerstetter *et al.* (1970) noted this potential and found that stimulation of $Na^+$ uptake (produced by increasing the external $Na^+$ concentrations) was correlated with a stimulation of acid efflux, rather than ammonia efflux. In addition, in this study, TEPs were monitored and shown to be insufficient to account for the increase in flux of either $Na^+$ or acid. Interestingly, the study by Kerstetter *et al.* (1970) demonstrates another drawback of whole-animal studies. They injected the carbonic anhydrase inhibitor acetazolamide into trout and found that both $Na^+$ uptake and acid efflux was inhibited. While these data could support the proposition that $Na^+/H^+$ exhange is present and limited by the production of protons by the hydration of $CO_2$ in the branchial cells, the fall in both fluxes could have been secondary to cardiovascular effects of the injected drug. That ammonia efflux did not change significantly during the same treatment argues against general cardiovascular effects, but it is possible that ammonia efflux is *via* a pathway which is relatively unaffected by cardiovascular changes. The unfortunate fact is that whole-animal studies do not allow the separation of cardiovascular from epithelial effects.

Thus, attempts to manipulate blood ionic concentrations, injection of potentially cardiovascular-active drugs, and lack of monitoring of the TEP present pitfalls which could bring into question the conclusions of many whole-animal studies. Some of these problems can be avoided by using externally applied drugs or by monitoring the efflux of an ionic species when ionic substitutions are made in the external medium. If one assumes that external addition of drugs or ionic substitutions do not have cardiovascular effects, and one monitors the TEP, many of the problems of earlier studies can be avoided. For example, we have recently found (Evans, 1977, 1982b; Evans *et al.*, 1979) that various species of marine teleost and elasmobranch fishes excrete ammonia and $H^+$, and that approximately 50% of the ammonia efflux and 100% of the $H^+$ efflux is dependent on external $Na^+$. Measurements of the TEP indicate that the coupling is not electrical. It is interesting to note that the excretion of ammonia from intact freshwater fishes is relatively unaffected by the removal of external $Na^+$ (deVooys, 1968; Kerstetter *et al.*, 1970; Maetz, 1973). Unfortunately, only Kerstetter *et al.* (1970) monitored the TEP, and they found that TEP changes were insufficient to account for any flux changes, or lack thereof.

The data from intact fish on $Cl^-/HCO_3^-$ exchange is more sparse, but less equivocal. Dejours (1969) found that when the external medium of a goldfish was changed from NaCl to $Na_2SO_4$, $CO_2$ excretion fell to zero and was restimulated when the fish was again placed into NaCl solutions. In addition, DeRenzis and Maetz (1973) demonstrated a good correlation between the uptake of $Cl^-$ and the net excretion of base by the goldfish, and DeRenzis (1975) found that addition of thiocyanate to the external bath inhibited $Cl^-$ uptake and base excretion. Unfortunately, only in the latter study were TEPs measured, but they could not account for the effect.

In most cases whole-animal studies treat the branchial transporting cells as "black boxes" and cannot separate events taking place on the basolateral *vs* apical surfaces of the cells. For example, the fact that removal of external $Na^+$ inhibits 50% or less of the ammonia efflux from marine fish supports the proposition (Evans, 1977) that $Na^+/NH_4^+$ exchange is taking place, but it does not, in itself, indicate the site of this ionic exchange system. Maetz and Garcia Romeu (1964) found that the carbonic anhydrase inhibitor acetazolamide inhibited both $Na^+$ and $Cl^-$ uptake when injected into the blood of the goldfish. They proposed that both $Na^+$ and $Cl^-$ uptake must therefore be limited by the production of $H^+$ (for the protonation of $NH_3$ which had been produced in the branchial cells) and $HCO_3^-$. Since $Na^+$ and $Cl^-$ are taken up independently from extremely low salinities (in micromolar ranges in some cases), and probably exchanged for these intracellular electrolytes (see above), it seems most appropriate to propose that the ionic exchange systems are on the apical border of the transporting cell (Maetz and Garcia Romeu, 1964; Kirschner, 1977, 1979). This model (Fig. 1) is supported by the finding that amiloride (which is known to inhibit uptake of $Na^+$ in a wide variety of tissues (Cuthbert *et al.*, 1979)) also inhibits both ammonia and acid efflux from fish (Kirschner *et al.*, 1973).

The potential problems of whole-animal studies are especially evident when one examines the history of the study of $Na^+$ and $Cl^-$ extrusion mechanisms in marine species. A more extensive discussion of this subject was presented elsewhere (Evans, 1979). In the late 1960's it was found that the marine teleost branchial epithelium contained significant quantities of the enzyme ($Na^+$-$K^+$ activated ATPase) which mediated $Na^+/K^+$ exchange in a variety of tissues, and that these enzyme activity levels were lower in freshwater species and after freshwater adaptation of euryhaline

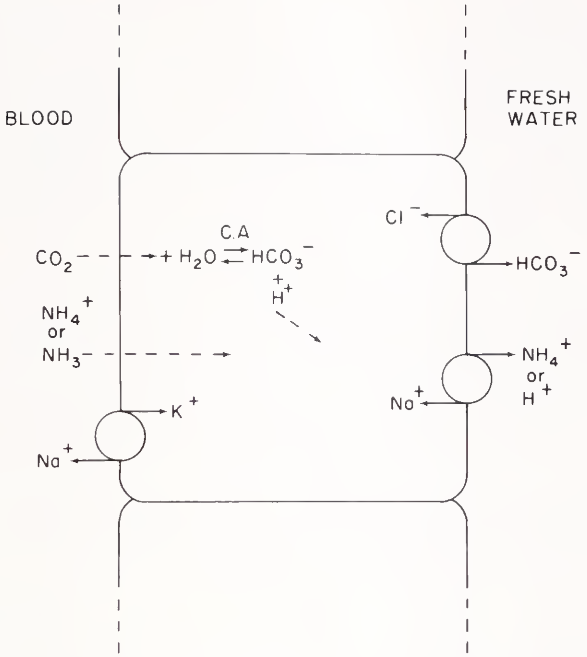

FIGURE 1. Tentative model for mechanisms of $Na^+$ and $Cl^-$ uptake by the branchial epithelium of freshwater fishes. Redrawn from Maetz and Garcia Romeu (1964). See text for details, supporting evidence, and additions.

species (Epstein *et al.*, 1967). Soon thereafter it was discovered that the efflux of radiosodium from the eel, *Anguilla anguilla*, was sensitive to the external (seawater) concentration of $K^+$ (Maetz, 1969). Thus, it appeared that the seawater fish gill, like so many other tissues, extruded unwanted $Na^+$ in exchange for seawater $K^+$, utilizing the enzyme $Na^+$-$K^+$ activated ATPase (Maetz, 1971).

This model was strengthened by the finding that another species of marine teleost (the fat sleeper, *Dormitator maculatus*) also possessed a $K^+$-sensive $Na^+$ efflux, with a $K^+$ sensitivity (delineated by the $K_m = 2$ m$M$ $K^+$) identical to that of the $Na^+$-$K^+$ activated ATPase extracted from the gill tissue (Evans *et al.*, 1973). In addition, the time course of activation of the enzyme was identical to the time course of activation of the $K^+$ sensitive $Na^+$ efflux when this species was transferred from fresh water to sea water (Evans and Mallery, 1975). However pleasant this model for $Na^+$ extrusion was, it rapidly became apparent that the system was much more complex. These initial studies neglected to measure the TEP during these ionic substitutions. Indeed, earlier studies (House, 1963; Evans, 1969) had demonstrated that the TEP across two species of marine teleosts was nearly identical to the equilibrium potential for $Na^+$, *i.e.*, $Na^+$ was possibly in passive equilibrium across the fish gill because the prevailing chemical gradient favoring net diffusional gain was balanced by a blood-positive (to sea water) electrical potential of sufficient magnitude to balance the chemical gradient. Thus, these data indicated that the net salt gain in sea water was not NaCl, but only $Cl^-$ because $Na^+$ was in electrochemical equilibrium. This idea has been at least partially substantiated by more recent whole-animal TEP determinations; however, it has been found that some

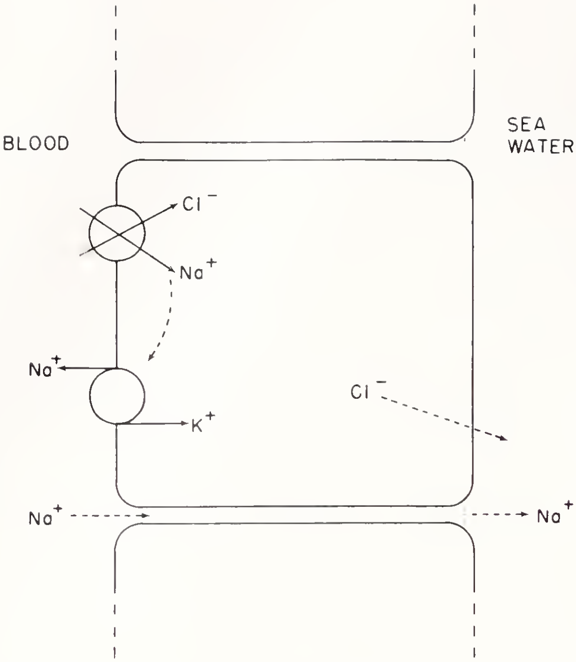

FIGURE 2. Current model for the mechanisms of $Na^+$ and $Cl^-$ extrusion by the branchial epithelium of marine fishes. Redrawn from Silva *et al.* (1977). See text for details and supporting evidence.

species apparently maintain TEPs distinctively below the equilibrium potential for $Na^+$ (see reviews by Kirschner, 1979, 1980, and Evans, 1980b). If $Na^+$ is in electrochemical equilibrium then the ionic substitution experiments which indicated that $Na^+/K^+$ exchange may be taking place may also possibly be explained by TEP changes. This has proved to be the case in some species (Potts and Eddy, 1973; Kirschner *et al.*, 1974) but not in others (Evans, 1975; Maetz and Pic, 1975; Evans and Cooper, 1976). In addition, $Na^+/Na^+$ exchange diffusion which was first described by Motais *et al.* (1966) has now been shown to be a TEP effect in some species (Potts and Eddy, 1973; Kirschner *et al.*, 1974) but not others (Evans, 1975; Maetz and Pic, 1975; Evans and Cooper, 1976).

Thus, whole-animal studies on the mechanisms for salt extrusion in sea water have left us with the rather unsatisfying conclusion that some animals may be extruding net amounts of $Na^+$ and $Cl^-$ and others may only need to extrude $Cl^-$ (the TEP of all marine teleosts examined to date is distinctly different from the equilibrium potential for $Cl^-$; Evans, 1980b). Studies of the mechanisms of $Cl^-$ extrusion by whole animals have indicated that it is sensitive to the external $K^+$ concentration (Epstein *et al.*, 1973) and to the external $HCO_3^-$ but not $OH^-$ concentration (Kormanik and Evans, 1979), and inhibited by injection of thiocyanate (Epstein *et al.*, 1973). In addition, both $Na^+$ and $Cl^-$ efflux are inhibited by injection of the $Na^+$-$K^+$ activated ATPase inhibitor ouabain into the blood of the eel, *Anguilla rostrata* (Silva *et al.*, 1977). Since it had been shown that $Na^+$-$K^+$ activated ATPase is actually located on the basolateral plasma membranes (Karnaky *et al.*, 1976), these authors proposed that, like many other tissues (Frizell *et al.*, 1979), the marine teleost gill epithelium secretes $Cl^-$ *via* a basolateral cotransport of $Na^+$ and $Cl^-$ (energized by the movement of $Na^+$ down its electrochemical gradient, which is maintained by $Na^+$-$K^+$ activated ATPase), followed by movement of $Cl^-$ down its electrochemical gradient from the cell to the sea water. $Na^+$ is maintained in electrochemical equilibrium. This model (Fig. 2) certainly goes far to explain most of the present data from intact animals, but, of course, does not explain $Na^+$ extrusion by fish which have been shown to maintain $Na^+$ out of electrochemical equilibrium (see review by Evans, 1980b). The sensitivity to external $HCO_3^-$ (Kormanik and Evans, 1979) is also not explained by this model.

Unfortunately, the use of intact animals precludes the most obvious experiments to test this interesting model. Ouabain is a potent cardiovascular agent and, indeed, Silva *et al.* (1977) did find that even the efflux of tritiated water from *A. rostrata* declined by some 40% after the injection of sufficient ouabain to produce a plasma concentration of $2.5 \times 10^{-6}$ *M*. This presumably represented some sort of alteration in blood flow through the branchial vasculature, which in theory could have had a more pronounced effect of the efflux of both $Na^+$ and $Cl^-$ than tritiated water. Thus, the fact that ouabain treatment inhibited $Na^+$ and $Cl^-$ efflux by 90% does not necessarily prove a direct effect on a basolateral uptake, dependent upon a functioning $Na^+/K^+$ exchange. In addition, one cannot specifically remove blood $Na^+$ to examine the effect on $Cl^-$ efflux (according to the Silva model, it would decline significantly).

In the past few years, whole-animal studies have been utilized to demonstrate that the $Na^+/H^+$ or $NH_4^+$ exchange which characterizes freshwater fish ion regulation is also present in marine species secondary to the needs of nitrogen and acid extrusion (see above). In fact we have found that the marine hagfish also possesses these ionic exchange systems (Evans, 1980a). Since hagfish have never entered fresh water (Hardisty, 1979) it appears that $Na^+/H^+$ or $NH_4^+$ exchange came about before the vertebrates entered fresh water, as an acid and nitrogen excretory device, rather than as an ionoregulatory device adaptive to freshwater existence. The presence of this system in marine species is therefore an indication of an ancient marine invention rather than a hold-over from a former existence in fresh water, as was formerly proposed (Evans, 1975).

Intact-animal studies have advanced our knowledge of fish branchial ion transport systems considerably in the past 20 years, but the limitations on the manipulation of intact animals has restricted the approaches to specific questions with somewhat limited answers. While the use of intact animals ensures (in theory) that proper perfusion and irrigation of the branchial epithelium is taking place, and that neural and hormonal inputs are present, it also ensures that substantial alterations in blood ionic components cannot be made, and that injection of known ionic transport inhibitors may induce secondary changes *via*, for instance, cardiovascular changes. Moreover, it does not allow one to separate transport steps at the basolateral *vs* apical borders of the transporting cells. In addition, the specter of stress with concomitant neuroendocrine changes is always present. For example, our finding that in both a marine teleost and marine elasmobranch replacement of the external sea water with $Na^+$-free artificial sea water (choline as the impermeant cation) resulted in cessation of net extrusion of $H^+$ and apparent extrusion of base leads us to believe that branchial $Cl^-/HCO_3^-$ exchange may be present, but usually "hidden" behind $Na^+/H^+$ exchange, especially under hypercapnic conditions (Evans, 1982b). We tested for $Cl^-/HCO_3^-$ exchange by injecting a bicarbonate load into both species with the expectation that we could stimulate net base excretion. However, in both cases (Evans, unpublished observations) we found that injection of a base load stimulated net $H^+$ extrusion rather than net base extrusion, secondary, presumably, to a stress response. In fact injection of only Ringer's solution results in a net efflux of $H^+$. Thus, the stress response (despite the use of anesthetic) complicates an investigation of $Cl^-/HCO_3^-$ exchange in intact animals.

Because of these problems, various *in vitro* approaches have been made to the study of fish gill transport in the past few years. We will start with perfusion of the head end since this technique was actually orginated in the 1930's.

## THE ISOLATED, PERFUSED HEAD PREPARATION

Keys (1931a) was the first to describe a fish preparation in which both the serosal and mucosal solutions bathing the branchial epithelium could be controlled. In this so-called "heart-gill" preparation (utilizing the eel, *Anguilla anguilla*) teleost Ringer's solution was perfused into the hepatic vein, and was pumped *via* the intact, beating heart to the gills. External irrigation of the gills with a small volume of fresh water was accomplished by pumping the water through a tube inserted into the mouth of the animal. Utilizing this preparation, the first *in vitro* experiments on branchial hemodynamic and active chloride transport mechanisms were described (Keys, 1931a,b; Bateman and Keys, 1932; Keys and Bateman, 1932; Keys and Wilmer, 1932). Thirty years later, the advent of isotopic tracers allowed a revitalization of the heart-gill preparation to attempt to define more clearly the NaCl movements across the gills (Tosteson *et al.*, 1962; Kirschner, 1969). The latter study modified the "heart-gill" preparation of the eel so that Ringer's was perfused into the ventral aorta *via* a pulsatile pump, thereby bypassing the heart. These pump-perfused gills appeared to be much more permeable to $Na^+$ than the heart-gill or *in vivo* systems also tested (Kirschner, 1969). By decreasing perfusate temperature, deterioration of ionic fluxes was reduced, but gill resistance still increased.

The utilization of the "heart-gill" preparation had one major undesirable attribute: the direct effects of various hemodynamic agents on the branchial vascular (*e.g.* epinephrine) could not be separated from their effects on the heart itself. The isolated, perfused head preparation (IPHP) of the trout, *Salmo gairdneri*, was developed by Payan and Matty (1975) and appeared to be much more viable as a tool for the study of osmoregulatory (and hemodynamic) parameters of the gills.

Briefly, the IPHP is prepared by decapitation of the fish posterior to the opercular openings after heparinizing and anesthetizing the animal. Cannulas are inserted into the ventral aorta proximal to the heart, and into the mouth. The preparation is then placed in a chamber which allowed the separation of irrigation fluid pumped over the gills from the efferent perfusate draining from the dorsal aorta and the open body cavity. Perfusion is accomplished either gravimetrically or by a peristaltic pump. Afferent flow rate or perfusion pressure is measured *via* a drop counter or pressure transducer connected to the perfusion line. In some preparations the dorsal aorta is cannulated, thus allowing the partitioning of the efferent perfusate into dorsal arterial and "venous" components (Girard and Payan, 1976; Claiborne and Evans, 1980).

This partitioning of respiratory and venous flows is possible since the blood leaving the respiratory lamellae in the gill may return *via* efferent filamental and branchial arteries to the dorsal aorta or be channelled through contractile anastomoses between the efferent filamental artery and the central venous sinus of the filament to the venous circulation (Fig. 3). In some species prelamellar anastomoses are also found (Boland and Olson, 1979) between the afferent filamental artery and the central vein of the filament, but these anastomoses are smaller and less

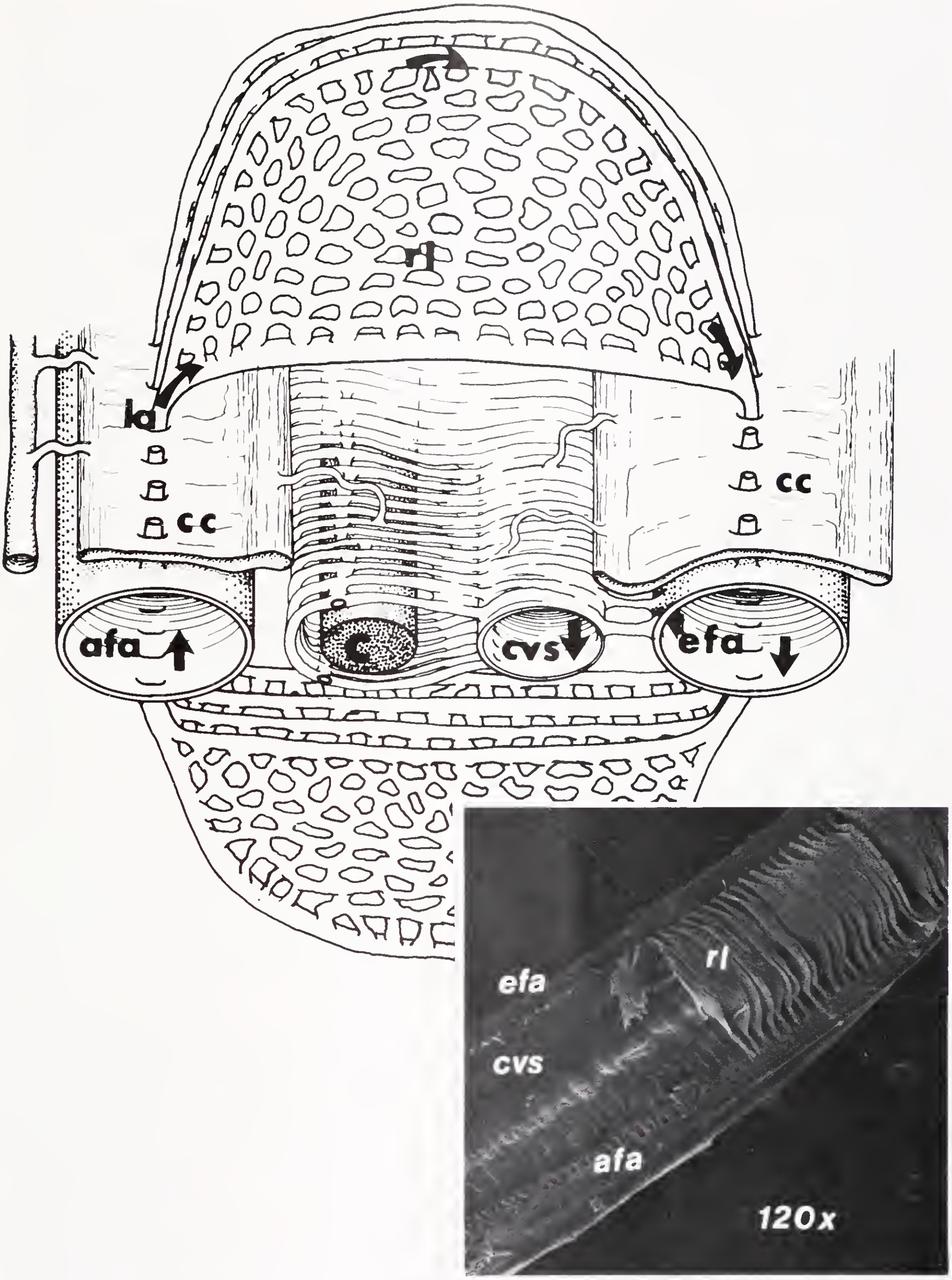

FIGURE 3. Filamental circulation of the gill of the snapper, *Lutjanus gresius*. Blood flows distally in the filament in the afferent filamental artery (afa) which leads to the respiratory lamellae (rl) *via* the lamellar arterioles (la). Blood leaves the lamellae in the efferent filamental artery (efa). Regular anastomoses between the efferent filamental artery and the central venous sinus (cvs) supply the extensive venous network surrounding the filamental cartilage (C). Companion nutritive vessels (cc) overlay both the afferent and efferent filamental arteries and connect to the central venous network *via* irregular anastomoses.

numerous than the postlamellar connections, and for that reason do not appear to form an effective bypass circuit around the lamellae (Farrell, 1980).

Since its inception, the IPHP has been used in a variety of investigations of gill hemodynamics (see Claiborne and Evans, 1980) and even brain metabolism (Callard *et al.*, 1981). We need concern ourselves only with the ionic transport studies.

Girard and Payan (1980) have recently reviewed their studies on the IPHP of the rainbow trout, *Salmo gairdneri*, which have demonstrated that the head is capable of carrying out $Na^+/NH_4^+$ exchange, both in fresh water (Payan, 1978) and in sea water (Payan and Girard, 1978). Unfortunately, no data have been published on the coupling of $Na^+$ influx to $H^+$ efflux or $Cl^-$ influx to $HCO_3^-$ efflux, despite the fact that both exchange systems have been described in the intact trout (Kerstetter *et al.*, 1970; Kerstetter and Kirschner, 1972). It is interesting to note that while Payan (1978) demonstrated a 1:1 stoichiometry for $Na^+$ and influx *vs* $NH_4^+$ efflux, a considerable ammonia efflux (approximately 70%) continued in the absence of $Na^+$ in the external bath, indicating clearly that the majority of the ammonia efflux is not coupled to external $Na^+$. These data corroborate our finding (Evans, 1977) that 50% or less of the ammonia efflux from intact marine species is dependent upon seawater $Na^+$. By examining the partitioning of the postlamellar (see Laurent and Dunel, 1980 for a review of fish gill morphology) perfusate flows into dorsal aorta *vs* "venous" flows, Girard and Payan (1977a) were able to demonstrate that all of the $Na^+$ and $Cl^-$ influx was across the lamellar epithelium, contrary to the situation in the perfused head of the seawater-adapted trout where a significant portion of the influx is across the filamental surfaces, presumably interlamellar (Girard and Payan, 1977b). Since this lamellar $Na^+$ influx in the freshwater-adapted head displays the characteristics of $Na^+/NH_4^+$ exchange (*i.e.* is sensitive to perfusate $NH_4^+$ concentrations; Payan, 1978) it appears that the active transport step for at least freshwater $Na^+$ balance is in the lamellar epithelium, rather than the so-called "chloride cells" of the filamental epithelium. This is the first, and only, evidence that we have that ionic extraction by freshwater fish may actually not involve the "Cl cells," which are generally thought to be the sites of active salt transport in both freshwater and seawater fish (see below). It is important to note that the influxes of both $Na^+$ and $Cl^-$ displayed by the perfused trout head were only 20–30% of those found *in vivo* (Girard and Payan, 1977a); however, $10^{-5}$ *M* epinephrine stimulated $Na^+$ influx to some 130% of *in vivo*, while the $Cl^-$ influx remained unchanged (Girard and Payan, 1977a).

The IPHP has also been utilized to examine more carefully the cellular localization of the $Na^+/NH_4^+$ exchange mechanism. Payan *et al.* (1975) demonstrated that addition of ouabain inhibited both $Na^+$ influx and ammonia efflux from perfused freshwater trout heads. However, Payan (1978) proposed that this inhibition was secondary to a primary, apical $Na^+/NH_4^+$ exchange which was sensitive to intracellular $Na^+$ concentrations (which were maintained by basolateral $Na^+$-$K^+$ ATPase). This model was based upon his finding that acetazolamide added to the perfusate inhibited ammonia clearance, as did amiloride added to the irrigation fluid (fresh water). More critically, he found that reducing the $NH_3$ concentration of the perfusate by approximately 10-fold (by reducing the pH by 1 pH unit) inhibited the excretion of ammonia by some 85%. It also inhibited sodium influx by about 60% (Payan, 1978). He therefore proposed that ammonia entered the cell as $NH_3$, was protonated *via* the hydration of $CO_2$ *via* carbonic anhydrase, and was excreted at the apical surface in exchange for $Na^+$ in the fresh water—the model first proposed by Maetz and Garcia Romeu in 1964 for intact fish (see above). We have recently approached the same problem with the IPHP of two marine teleost fishes (*Myoxocephalus octodecimspinosus*, the longhorned sculpin; and *Opsanus beta*, the gulf toadfish) and found that increasing the perfusate $NH_3$ concentration (by increasing the pH) did not stimulate ammonia efflux; however, increasing only the $NH_4^+$ concentration (by increasing the ammonia concentration, while reducing the pH) stimulated the ammonia efflux significantly (Goldstein *et al.*, 1982). Since only approximately 50% of the ammonia efflux is coupled to $Na^+$ in intact marine fishes (see above), it appears that a significant component of ammonia efflux from at least marine fish gills is *via* diffusion of $NH_4^+$ across the branchial epithelium. This may be *via* leaky "tight junctions" since the marine teleost gill has been shown to be quite leaky to cations and even large organic molecules (Karnaky, 1980). The proposition that $NH_4^+$ can diffuse across the marine teleost gill is supported by our earlier finding that addition of 200 m*M* $NH_4Cl$ solutions to Na-free artificial sea water depolarized the TEP across the intact toadfish to the same extent as 200 m*M* NaCl (Evans, 1977). It is important to note that the ammonia efflux from the IPHP of both *O. beta* and *M. octodecimspinosus* is close to that found *in vivo* (Goldstein *et al.*, 1982). We have found that ouabain added to the Ringer's solution (containing 1 m*M* $NH_4Cl$) perfusing the IPHP of *O. beta* inhibited ammonia efflux by some 50%. This could have been an indirect effect (as proposed by Payan, 1978); however, we have also found that addition of $K^+$ to the perfusate inhibited ammonia efflux, indicating a direct interaction at the basolateral border. In addition, we found that neither ouabain nor $K^+$ produced hemodynamic effects sufficient to account for the observed inhibition of ammonia efflux (Claiborne *et al.*, 1982). We conclude that, at least in this species, $Na^+/NH_4^+$ exchange is basolateral, rather than apical, and running through the $Na^+$-$K^+$ activated ATPase. The $NH_4^+$ sensitivity of this enzyme is well documented (see below).

Since intact marine teleosts and elasmobranches have been shown to excrete $H^+$ in exchange for $Na^+$ (Evans *et al.*, 1979; Evans, 1982b) it would be of great interest to use an IPHP to examine this system in greater detail.

Girard (1976) used the IPHP of the seawater-adapted trout to examine various aspects of the extrusion of $Na^+$ and $Cl^-$. He found that the effluxes of $Na^+$ is near to that measured *in vivo* and that effluxes of both $Na^+$ and $Cl^-$ were stimulated by addition of $K^+$ to the external medium; unfortunately he did not report TEPs so that one could separate chemical *vs* electrical coupling. Claiborne and Evans (1981) have recently shown that the IPHP of *M. octodecimspinosus* maintains a $Na^+$ efflux near *in vivo* levels, but a $Cl^-$ efflux significantly below that found in the intact fish. The efflux of neither ion is affected by large alterations in the irrigation rate, but changes in perfusion rate (and therefore pressure) produce significant alteration in the $Na^+$ efflux, with no effect on the $Cl^-$ efflux. This argues for separate pathways for the bulk of the $Na^+$ *vs* $Cl^-$ efflux which supports the extrusion model of Silva *et al.* (1977; see above), but does not support the recent porposition (Sargent *et al.*, 1978; Kelly *et al.*, 1981) that NaCl is forced across the leaky "tight junctions" of the branchial epithelium by arterial blood presure.

Various direct tests of the "Silva model" are theoretically possible with the IPHP. Since the perfusate can be manipulated it would be of great interest to test the sensitivity of the $Cl^-$ efflux to removal of $Na^+$ from the perfusate. This would be the most direct test of the proposed co-transport of $Na^+$ and $Cl^-$ which is the core of this model. Unfortunately, the branchial vasculature of at least *M. octodecimspinosus* is quite sensitive to the choline used to replace the perfusate $Na^+$ and subsequent large increases in afferent pressure and $Cl^-$ efflux obscure any changes in $Cl^-$ efflux which may have been produced by the lack of $Na^+$ (Claiborne and Evans, unpublished).

Kelly *et al.* (1981) have recently found that $10^{-4}$ *M* ouabain inhibits both the $Na^+$ and $Cl^-$ efflux from the IPHP of the eel (*Anguilla anguilla*) by some 30–40% with no effect on the afferent perfusion pressure, or the efflux of tritiated water. Thus, in these experiments, one can be rather certain that the effect of ouabain was a direct one on some component of the transport system, rather than an indirect effect through hemodynamic changes.

To date, no report of a transepithelial potential measured across the gills of an IPHP has appeared in the literature. TEP changes across the branchial epithelium must be monitored concurrent with ion substitution or drug inhibition experiments (see above). Recently, we have found it possible to measure the TEP across the gills of the IPHP of *M. octodecimspinosus* in sea water. We found that the IPHP TEP was similar to that measured *in vivo*. Substitution of $Na^+$ or $Cl^-$ with the appropriate impermeant ion in the external sea water resulted in large depolarizations when $Na^+$ was replaced, but no alterations were observed after $Cl^-$ substitutions (Claiborne and Evans, 1981). These responses, observed both *in vivo* and *in vitro*, indicate that the gills of the IPHP (and the sculpin *in vivo*) are more permeable to $Na^+$ than to $Cl^-$, as shown in many other teleosts which possess a positive TEP (Evans, 1979).

While the IPHP enables the investigator to ask questions impossible using intact systems, it still presents some limitations. The majority of the studies of ion transport by the IPHP have utilized the trout head, which suffers from rather serious hemodynamic degradation in a short period of time. Girard (1976) found that the gill resistance increased by some 5-fold within 30 minutes and Wood (1974) found that relatively linear and stable pressure *vs* flow relationships were only possible if post-branchial efferent pressures were maintained by a column of irrigation solutions. To delay the hemodynamic degradation of the trout head, epinephrine has sometimes been added to the perfusate (Payan, 1978). However, it is clear that this hormone stimulates $Na^+$ uptake in fresh water and inhibits it in sea water (Girard, 1976; Payan, 1978; Shuttleworth, 1978). This hemodynamic degradation of the IPHP may be species specific since we have recently found that IPHPs of the sculpin, toadfish, and shark "pup" (*Squalus acanthias*) can maintain relatively consistant gill resistances for 3–8 hours (Claiborne and Evans, 1980; Oduleye *et al.*, unpublished results; Evans and Claiborne, 1982). In all three species the afferent pressures are at *in vivo* levels when the perfusion rate is in the same range as the *in vivo* cardiac output, despite the fact that postbranchial efferent resistances are near zero. It is obvious that other species should be examined.

Importantly, most of the IPHP studied to date maintain $Na^+$ and/or $Cl^-$ fluxes significantly below *in vivo* levels (see above). In fact, in a recent study using the IPHP of *A. anguilla* the measured $Na^+$ and $Cl^-$ fluxes were only 10% of the fluxes measured *in vivo* (compare Kelly *et al.*, 1981, with Epstein *et al.*, 1973). Whether the reduced effluxes found in some species are secondary to incomplete perfusion of the branchial vasculature or lack of stimulatory hormones normally found *in vivo* remains to be determined.

Probably the most important, and least often controlled, parameter of the IPHP is the ratio of the perfusate inflow to outflow. Most authors do not note this comparison which is a direct measure of the structural/hemodynamic integrity of the system. It should be obvious that even slight leakage of the perfusate either into the external medium or the head tissues will produce quite spurious determination of ion flux rates. These leak pathways may not affect active pathways, but they may obscure the latter's importance or even presence in the total unidirectional flux as determined with radioisotopes. Losses of up to 30% of the perfusate during its transit of the gills has been reported by some investigators in personal communications. Again this may be species specific because we have found that the sculpin, toadfish, and dogfish shark "pup" maintain inflow:outflow ratios of approximately 1.0 (Claiborne and Evans, 1980; Oduleye *et al.*, unpublished results; Evans and Claiborne, 1982). In summary, present data indicate that the isolated, perfused head preparation may allow a more critical dissection of the mechanisms of NaCl transport by the fish branchial epithelium than is possible with *in vivo* studies. It is important to note that in most instances published IPHP studies have corroborated the findings of earlier studies using intact animals, despite the fact that many of the preparations (especially those utilizing the trout head) display significant degeneration of the hemodynamics of the branchial vasculature. It is clear that more species need to be investigated and that greater attention be paid to the ratio of the inflows:outflows and the TEPs maintained by the IPHP.

## THE ISOLATED, PERFUSED GILL PREPARATION

An alternative to the perfused head is the isolated, perfused gill, which has been used rather extensively in the past 15 years. Although methods vary slightly, generally isolated gills are prepared by initial perfusion of an anesthetized animal with Ringer's solution. When filaments are free of blood, individual arches are selected and removed. The afferent and efferent branchial arteries are cannulated, and the arch is placed in a well-stirred external bath. In early work a constant pressure reservoir provided afferent pressure, but more recently pulsatile flow generated by a pump has been employed. Efferent pressure is set by the height of the efferent cannula above the preparation.

Like the perfused head, the perfused gill preparation has been used extensively to investigate the hemodynamics of branchial circulation, but rather little to study gill ion transport. To a considerable extent this is apparently due to the isolated gill's ability to maintain reasonable hemodynamics (*e.g.* Bergman *et al.*, 1974; Holbert *et al.*, 1979) but inability to maintain proper irrigation. Unfortunately, even vigorous stirring of the irrigation bath apparently does not mimic the irrigation patterns found in the intact animal, or the perfused head. For example, Shuttleworth and Freeman (1974) described $Na^+$ and $Cl^-$ effluxes from the perfused eel (*Anguilla dieffenbachii*) gills that were only 10–15% of those found in the intact fish, and Farmer and Evans (1981) have recently found that the efflux of $Cl^-$ from perfused pinfish (*Lagodon rhomboides*) gills is 45% that of the intact fish. Nevertheless, the perfused gill has provided us with some information unavailable with other techniques. Shuttleworth *et al.* (1974) demonstrated that the TEP across the perfused marine flounder (*Platichthyes flesus*) gill was approximately 7 mV inside positive when the gill was perfused and irrigated with Ringer's solution. Addition of ouabain inhibited the TEP, indicating that salt extrusion was electrogenic, and that $Na^+$-$K^+$ activated ATPase played an important role. The finding of a substantial TEP when no chemical gradients existed across the gill epithelium demonstrated that the TEP across intact marine fish was probably a combination of electrogenic transport and differential ionic permeabilities. Studies with intact marine fish had suggested that the TEP was primarily the result of a much higher cation than anion permeability (Potts and Eddy, 1973; Kirschner *et al.*, 1974). More recent studies have lent support for the "Silva model" for coupled $Na^+$ and $Cl^-$ transport by the gill epithelium. Farmer and Evans (1981) have shown that the $Cl^-$ efflux from the perfused pinfish gill is inhibited by removal of $Na^+$ from the perfusate, or addition of furosemide. Furosemide has been found to inhibit coupled $Na^+$ and $Cl^-$ transport in a wide variety of epithelial tissues (Frizell *et al.*, 1979).

The perfused gill has been utilized to examine salt uptake by freshwater fish. Richards and Fromm (1970) found that addition of ouabain to the Ringer's solution perfusing the isolated trout gill inhibited the uptake of $Na^+$ and Shuttleworth and Freeman (1974) found that removal of $K^+$ from the perfusate inhibited $Na^+$ uptake by the eel gill. Both studies support the conclusion that basolateral $Na^+/K^+$ exchange (mediated *via* $Na^+$-$K^+$ activated ATPase) plays a role in $Na^+$ uptake in fresh water.

## THE ISOLATED OPERCULAR EPITHELIUM

An extensive literature indicates that the mitochondria-rich "chloride cell" of the fish gill epithelium plays an important role in osmoregulation (for an extensive review see *The Biology of the Chloride Cell*: Jean Maetz Memorial Symposium, *American Journal of Physiology* **238:** R141–R276, 1980). Quite recently a technique has been developed which has enabled a much more direct study of the biophysics of ion transport across this cell than has been possible with intact fish, or isolated heads or gills.

Burns and Copeland (1950) demonstrated that "chloride cells" are widely distributed throughout the head region of the killifish, *Fundulus heteroclitus*, but it was not until 1977 that it was shown that the opercular epithelium of this species possesses a cellular population which is 50–70% "chloride cells" whose cytology and ultrastructure is identical to the "chloride cells" in the gill epithelium (Fig. 4) (Karnaky *et al.*, 1976; Karnaky and Kinter, 1977). Thus, the opercular epithelium presented the unique opportunity to investigate the function of "chloride cells" on a flat epithelium, rather than on the extremely complex branchial epithelium. A flat epithelium can be dissected free and mounted in an "Ussing Chamber" which enables a strict thermodynamic approach to the electrical and chemical events of ion transport. In this way one can carefully control the ionic composition of both serosal and mucosal solutions bathing the tissue and measure net movements of ions quite accurately. In addition, any spontaneously generated electrical potentials can be measured and nulled (to quantify the short-circuit current), and resistances can be calculated. Since the original description of the opercular epithelium of *F. heteroclitus*, similar, "chloride cell"-rich tissues have been found in the operculum of *F. grandis* (Krasny and Evans, 1980) and *Sarotherodon mossambicus* (Foskett *et al.*, 1979) as well as the jaw epithelium of *Gillichthys mirabilis* (Marshall and Bern, 1980).

When the isolated opercular epithelium from seawater-adapted killifish is bathed bilaterally with a Ringer's solution having an ionic composition similar to *F. heteroclitus* plasma, a potential difference oriented serosa (blood) positive is generated (Degnan *et al.*, 1977; Karnaky *et al.*, 1977). Subsequent isotopic flux studies (Table I) showed that this potential difference was the result of the net transport of $Cl^-$ outwards across the tissue, *i.e.* blood side to seawater side; there was no net transport of $Na^+$ across the epithelium (Degnan *et al.*, 1977; Karnaky *et al.*, 1977). These were the first unequivocal studies showing that killifish maintain ionic homeostasis in sea water by actively extruding chloride into the external milieu. Equivalancy between the short-circuit current and net $Cl^-$ secretion has also been observed in the "chloride cell" containing opercular epithelia of *F. grandis*

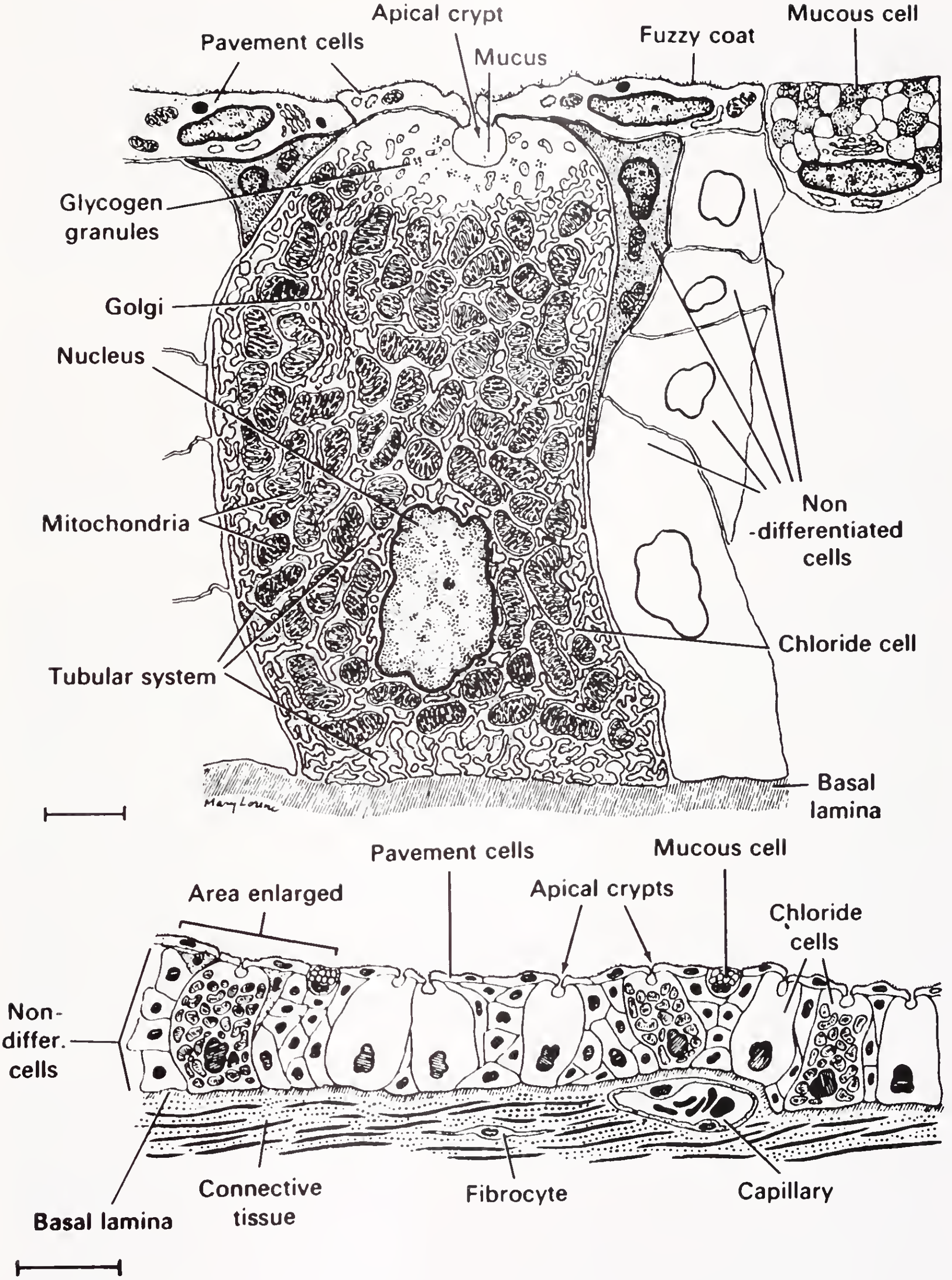
Apical crypt
Pavement cells
Mucus
Fuzzy coat
Mucous cell
Glycogen granules
Golgi
Nucleus
Mitochondria
Tubular system
Non -differentiated cells
Chloride cell
Basal lamina
Pavement cells
Mucous cell
Area enlarged
Apical crypts
Chloride cells
Non-differ. cells
Basal lamina
Connective tissue
Fibrocyte
Capillary

FIGURE 4. Schematic of the ultrastructure of a "chloride cell" (upper) and opercular epithelium (lower) from the opercular epithelium from *Fundulus heteroclitus*. In this tissue 50–70% of the cellular population is represented by "chloride cells" whose cytology is identical to that described for the branchial epithelium of teleosts. Reproduced with kind permission from Degnan *et al.* (1977). See text for details of the physiology of this opercular tissue. Scale is 20 $\mu$m.

TABLE I

*Isotopic fluxes and electrical properties across the short-circuited opercular epithelia of seawater-adapted* Fundulus heteroclitus *gassed with 95% oxygen, 5% carbon dioxide.*

| | Efflux | Influx | Net Flux | SCC | PD |
|---|---|---|---|---|---|
| Cl | 7.23 ± 2.13 | 2.86 ± 1.13 | 4.46 ± 1.09/119.6 ± 29.3 | 119.2 ± 22.9 | 12.6 ± 1.2 |
| Na | 2.63 ± 0.45 | 2.95 ± 0.26 | −0.32 ± 0.62/−8.6 ± 16.5 | 74.4 ± 10.3 | 10.1 ± 1.5 |

N for fluxes is 8, N for electrical properties is 16. Fluxes in $\mu Eq \cdot cm^{-2} \cdot h^{-1}$, net fluxes in $\mu Eq \cdot cm^{-2} \cdot h^{-1}/\mu A \cdot cm^{-2}$. Short circuit current (SCC) in $\mu M \cdot cm^{-2}$ and potential difference (PD) in mV, serosa relative to mucosa. Data from Degnan *et al.* (1977). Note that the SCC is identical to the net influx of Cl with no net movements of Na.

(Krasny, 1981) and *Sarotherodon mossambicus* (Foskett *et al.*, 1979) and jaw epithelium of *Gillichthys mirabilis* (Marshall and Bern, 1980).

The transport mechanisms in "chloride cells" for chloride appears very similar to that found in most chloride-transporting epithelial types (Frizzell *et al.*, 1979; Frizzell and Duffey, 1980). Namely, $Cl^-$ efflux is dependent upon the presence of $Na^+$ in the serosal medium (Degnan and Zadunaisky, 1980a, 1981; Mayer-Gostan and Maetz, 1980) and is blocked by the transport inhibitors furosemide or ouabain on the serosal side (Degnan *et al.*, 1977; Karnaky *et al.*, 1977; Mayer-Gostan and Maetz, 1980).

Studies utilizing the short-circuit current techniques made in conjunction with fluorescence microscopy techniques have provided direct evidence that the "chloride cell" is the "active" ionocyte involved in seawater teleost osmoregulation. DASPMI, a low toxicity, specific fluorescent stain for mitochondria in living cells (Bereiter-Hahn, 1976), has been used to stain "chloride cells" in the opercular epithelium of *F. heteroclitus* (Zadunaisky, 1979). Studies using this dye in the opercular epithelium of *F. heteroclitus* (Karnaky *et al.*, 1979) and the jaw skin epithelium of *Gillichthys mirabilis* (Marshall and Nishioka, 1980) have shown a linear correlation between "chloride cell" density and the magnitude of the short-circuit current. Similarly, Foskett *et al.* (1979) have shown that the increase in "chloride cell" density and size is correlated to the development of a short-circuit current in the opercular epithelium of *Sarotherodon* when the fish is acclimated to sea water. More recently, Foskett and Scheffey (1982) have found, using a vibrating probe technique, that current generated by the short-circuited opercular epithelium is directly over the "chloride cells". This is certainly the most definitive demonstration that the "chloride cells" are the site of electrogenic $Cl^-$ transport across the fish branchial epithelium.

By studying the voltage dependency of the unidirectional flux of an ion across an epithelium one can predict the nature (conductive *versus* electroneutral) and the pathway (cellular *versus* paracellular) of ion flow (Frizzell and Schultz, 1972; Mandel and Curran, 1972). Results from studies made in opercular epithelia of *F. heteroclitus* (Degnan and Zadunaisky, 1980b) and *F. grandis* (Krasny, 1981) indicated that there were no significant differences between the predicted and measured fluxes for either the efflux or influx of $Na^+$, thus allowing the conclusion that the $Na^+$ fluxes in opercular epithelia are passive and traverse only one rate-limiting barrier. This rate-limiting barrier presumably is represented by the tight junctional complex between neighboring "chloride cell" (Sardet *et al.*, 1979; Ernst *et al.*, 1980) as is indicated from experiments with triaminopyrimidine (TAP). TAP, which blocks passive cation transport through the paracellular pathway in "leaky" epithelia (Moreno, 1975) reduces the $Na^+$ efflux 84.1%, while reducing the total tissue conductance 77%, in the opercular epithelium of *F. heteroclitus* (Degnan and Zadunaisky, 1980b).

These results suggesting passive $Na^+$ movements as well as the results from studies made on the mechanism of chloride secretion in the opercular epithelium have provided strong direct evidence for the Silva *et al.* (1977) model for "chloride cell" function in seawater teleosts.

Whereas the isolated opercular epithelium from seawater-adapted teleosts has been used to define the ion transport properties of "chloride cells," the use of this preparation in the study of freshwater ion regulation is relatively uninvestigated. Although intact *F. heteroclitus* maintain ionic homeostasis in fresh water by extracting $Na^+$ and $Cl^-$ from the environment (Maetz *et al.*, 1967; Potts and Evans, 1967), opercular epithelia from freshwater-adapted *F. heteroclitus* continue to secrete $Cl^-$ (Degnan *et al.*, 1977). This may, in fact, be due to autoregulation of the apical membrane permeability to $Cl^-$ induced by the exposure of freshwater opercular epithelia to a Ringer bathing media containing 142.5 m*M* Cl, *i.e.* chloride regulates its own membrane permeability (Ques-von Petery *et al.*, 1978). Evidence for this supposition can be found in experiments performed on seawater-adapted opercular epithelia where removal of $Cl^-$ from the mucosal bathing media results in a decrease in tissue conductance and reduces the rate of $Cl^-$ secretion (Degnan and Zadunaisky, 1980a). On the other hand, opercular epithelia, isolated from normally freshwater-occurring *Sarotherodon* (Foskett *et al.*, 1979) or from *F. heteroclitus* which had been chronically injected with the "freshwater" hormone prolactin (Mayer-Gostan and Zadunaisky, 1978), are characterized by low short-circuit currents and high electrical resistances. This might be expected since: 1) the active chloride secretory process is "turned off;" and 2) the proposed ionic uptake mechanisms for both $Na^+$ and $Cl^-$ in the branchial epithelium of freshwater teleosts are, in fact, one for one electroneutral (electrically silent) exchanges: $Na^+/H^+$ and/or $NH_4^+$ and $Cl^-/HCO_3^-$ (see above).

Although it has been suggested that the $Na^+/H^+$ or $Na^+/NH_4^+$ exchanger may be located in the pavement cells of the lamellae of the branchial epithelium (Girard and Payan, 1980), these cells are derived from the filamental epithelium (Morgan, 1974; Laurent and Dunel, 1980) and are identical to the pavement cells of the opercular epithelium as determined by thin section electron microscopy (Karnaky and Kinter, 1977; Ernst *et al.*, 1980) and freeze-fracture (Sardet *et al.*, 1979; Ernst *et al.*, 1980) techniques. Thus, the use of the isolated opercular epithelial preparation with the pH-stat technique may yield new and important information concerning the ionic mechanisms involved in acid-base balance in both freshwater and seawater teleosts.

In theory, the isolated opercular epithelium may provide us with a vehicle for studying intracellular ionic concentrations and basolateral *vs* apical transport events *via* microelectrodes, in a manner similar to that recently used for a variety of transporting epithelia (Frizell *et al.*, 1979). However, the complex geometry of the extensive basolateral tubular invaginations results in a relatively sparse cytoplasm which may hinder such determinations.

It is obvious that the isolated opercular epithelium has allowed substantial advances in the investigation of the biophysics of NaCl extrusion by a seawater-acclimated teleost. However, one must be cautious when extending these data to all marine teleosts, and especially those species which seem to maintain $Na^+$ out of electrochemical equilibrium. In addition, it remains to be seen if it will be useful for the investigation of other transport events such as $Na^+/NH_4^+$, $Na^+/H^+$, and $Cl^-/HCO_3$ exchange.

## ISOLATION AND CHARACTERIZATION OF TRANSPORT ATPASES

The foregoing demonstrates the central role of $Na^+$-$K^+$ activated ATPase in ion balance and nitrogen excretion by the teleost branchial epithelium. This subject has also been recently reviewed by Epstein *et al.* (1980), Karnaky (1980), and Towle (1981). The assay of enzymatic activity primarily in whole gill homogenates has been especially productive in assessing salinity adaptive changes. This approach may be biased by differences between biochemical techniques (homogenization time, temperature, pH detergents, *etc.*) of different laboratories, and changes in tissue protein levels which will bias specific activity measurements if microsomal fractions are used. Unfortunately, the biochemical isolation and characterization of this presumptive transport enzyme is rather rare. Isolation and purification is certainly the approach which ought to be more productive in defining the ionic parameters that this gill enzyme functions under.

Partial characterizations of branchial $Na^+$-$K^+$ activated ATPase have been published (Kamiya and Utida, 1969; Pfeiler and Kirschner, 1972; Giles and Vanstone, 1976; Ho and Chan, 1980), but the publications from Sargent's laboratory (Sargent and Thomson, 1974; Bell *et al.*, 1977; Bell and Sargent, 1979; Sargent *et al.*, 1980) present the most detailed analysis of the enzyme from the fish gill (the Atlantic eel, *Anguilla anguilla*). They have purified the $Na^+$-$K^+$ activated ATPase to a specific activity of approximately 400 $\mu M \cdot \text{mg protein}^{-1} \cdot h^{-1}$, one to two orders of magnitude greater than that described by other authors (see Kirschner, 1980 for representative data). The enzyme, like that isolated from mammalian kidney and shark rectal gland (Dahl and Hokin, 1974; Schwartz *et al.*, 1975) is phosphorylated in the presence of $Na^+$ and Mg·ATP to produce a phosphoenzyme intermediate, which is dephosphorylated in the presence of $K^+$. Ouabain inhibits the dephosphorylation step and other cations, including $NH_4^+$, can substitute for $K^+$ at the dephosphorylation step, with varying affinities. Bell *et al.* (1977) found that the affinity of the purified enzyme for $NH_4^+$ was slightly less than for $K^+$, while Mallery (1979) found that partially purified enzyme from *O. beta* displayed a higher affinity for $NH_4^+$ than for $K^+$. It is interesting to note that this species displays a ouabain and $K^+$-sensitive ammonia efflux (Claiborne *et al.*, 1982). Unfortunately we have no data on the molecular weight or subunit structure of the fish branchial $Na^+$-$K^+$ activated ATPase.

We know even less about a putative anionic transport ATPase. Kerstetter and Kirschner (1974) described an ATPase fraction from trout branchial tissue which was stimulated by $HCO_3^-$ and inhibited by thiocyanate. Both the enzyme and $Cl^-$ influx was inhibited by thiocynate, which is especially surprising considering that the enzyme was inhibited, rather than stimulated by the addition of $Cl^-$ to the incubation medium. Importantly, comparison with succinic dehydrogenase activity as a mitochondrial marker indicated that the $HCO_3^-$-stimulated ATPase was in both mitochondrial and microsomal fractions. More recently DeRenzis and Bornancin (1977) and Bornancin *et al.* (1980) have described a microsomal ATPase which is stimulated by both $HCO_3^-$ and $Cl^-$, and inhibited by thiocyanate. Importantly, they have shown that this fraction is not contaminated by mitochondrial anion ATPase. They suggest that the enzyme is important in $Cl^-$ balance and acid/base regulation in fresh water since previous studies (DeRenzis, 1975) had shown that $Cl^-$ influx was correlated with base (presumably $HCO_3^-$) excretion and inhibited by thiocyanate (see above). It is unclear if the enzyme functions in $Cl^-$ transport in the marine teleosts. Kormanik and Evans (1979) have described an external $HCO_3^-$-sensitive efflux of $Cl^-$ from *O. beta* in sea water, and Epstein *et* *al.* (1973) did find that injection of thiocyanate inhibited $Cl^-$ efflux from seawater eels. However, the fact that the activity of the $Cl^-$-$HCO_3^-$ activated ATPase did not change upon acclimation to sea water (Kerstetter and Kirschner, 1974; Bornancin *et al.*, 1980), despite a significant difference in the rate of $Cl^-$ transport across the freshwater *vs* seawater gill (Evans, 1979) suggests that its major role may be in the freshwater environment. Indeed, the Silva model for NaCl extrusion by marine teleosts (Silva *et al.*, 1977) suggests that $Cl^-$ exits the Cl cell down electrochemical gradients across the apical surface of the "chloride cell," rather than *via* $Cl^-/HCO_3^-$ exchange. The latter cannot be ruled out at present however. It is clear that a more detailed investigation of the role of $Cl^-$-$HCO_3^-$ activated ATPase in fish ion regulation is needed.

## THE ELASMOBRANCHS

Interest in the rectal gland has nearly stifled investigation of the elasmobranch branchial epithelium. However, the ability of some species to tolerate sea water for prolonged periods after removal of the rectal gland, and the recent finding that $Na^+/NH_4^+$ and $Na^+/H^+$ ionic exchanges are resident in the elasmobranch branchial epithelium (Evans, *et al.*, 1979; Evans, 1982b) suggest that the gills may play some role in salt extrusion (see Evans, 1979 for a more complete discussion of the role of the rectal gland *vs* branchial epithelium).

To a considerable extent the paucity of data on the mechanisms of ionic transport across the elasmobranch gill is secondary to their relatively large size, disposition, and characteristic extremely low ionic fluxes (Evans, 1979). Nevertheless the few published measurements of the TEP indicate that both Na and Cl are maintained out of electrochemical equilibrium (Evans, 1980). We have recently found that prenatal "pups" of the spiny dogfish (*Squalus acanthias*) are plentiful and easy to handle and display the hallmarks of adult elasmobranch osmoregulation (Kormanik and Evans, 1978; Evans and Mansberger, 1979; Evans and Oikari, 1980). Importantly we have found that the head can be easily perfused and that it maintains hemodynamic stability for 2–3 hours (Evans and Claiborne, 1982). It is hoped that this preparation will allow a more careful dissection of any salt transport mechanisms which may reside in the elasmobranch branchial epithelium.

## CONCLUSIONS

It should be obvious from this rather cursory review that substantial strides have been made in the elucidation of the transport parameters of the fish gill, due in no small part to the use of various "pieces" of the whole animal. To a considerable extent the techniques have been complementary with data from one system corroborating, but expanding, data from another system. However, each of the approaches has its advantages and disadvantages, which must be appreciated and accounted for. It is also obvious that, per usual, the number of species of fishes which have been examined is vanishingly small, and no single species has been examined utilizing all of the techniques described in this review. It is therefore appropriate to suggest that more species should be examined and that more investigators should use a variety of techniques, rather than a single method of approach.

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# THE ANATOMY AND FINE STRUCTURE OF THE EYE IN FISH. VI CILIARY TYPE TISSUE IN NINE SPECIES OF TELEOSTS

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## ABSTRACT

The eyes of teleost fishes do not have ciliary bodies. Therefore there is no ciliary epithelium *per se*, the tissue normally assumed to secrete aqueous humor. When examined at the electron microscope level a layer of nonpigmented cells on the back of the fish iris shows many similarities to the ciliary epithelium of mammals. The tissue of fish iris has strategically located zonulae occludents similar to those forming the blood-aqueous barrier in mammals. There is a marked lateral interdigitation of cells as seen in mammalian ciliary tissue and as seen in the specific salt absorbing cells found in the gills of brackish water adapted crabs. The teleost tissue also has numerous intercellular spaces (ciliary channels?) distributed in the same fashion as in mammalian ciliary epithelium. Although there is no morphological evidence for the secretion of aqueous humor, there is indirect evidence that the nonpigmented cells absorb salt to produce the hypotonic aqueous humor that is unique to teleosts.

## INTRODUCTION

The morphology of the cells or tissue which secretes aqueous humor in the cold blooded vertebrates has received comparatively little attention. Fish have been almost completely neglected. They present an interesting problem in that their eyes completely lack the ciliary muscle and associated epithelium. Instead, the lens is suspended by a membrane dorsally and anchored ventrally by a hillock of muscle (campanula of Halleri). The muscle retracts the lens to accomplish accommodation.

Zadunaisky (1972, 1973) has studied the electrolyte content of the aqueous humor in several fishes and has also made preliminary observations on the possible site of secretory origin. The epithelium on the posterior surface of the iris proves to be the likely source of the primary aqueous humor.

There is universal agreement that in the higher vertebrates the ciliary body in some manner secretes the aqueous humor. The secreted fluid then passes through the pupil to the anterior chamber and exits via the trabecular meshwork and the canal of Schlemm. Evidence indicates that the secretion is accomplished by active transport.

The fine structure of the ciliary body and its epithelium in mammals has been thoroughly investigated. To name a few investigators: Pappas and Smelser (1961); Pappas and Tennyson (1962); Tormey (1963, 1964); Kaye and Pappas (1965); Bairati and Orzalesi (1966); Smith (1971); Raviola (1971, 1974); Shabo and Maxwell (1972, 1973); Uusitalo *et al.* (1973); Okisaka (1976a, b); Hara *et al.* (1977).

For reviews of the comparative composition of aqueous humor and the release of aqueous humor see Cole (1974), and Tripathi (1974), respectively.

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Abbreviations: NPL, nonpigmented layer; PL, pigmented layer.

The following report is an expansion of Zadunaisky's initial studies and utilizes a wider range of species and different electron microscopy techniques. It also is a continuation of my own investigations of the eyes of fish (Copeland, 1974a,b, 1976, 1980; Copeland and Fitzjarrell, 1975; Copeland and Brown, 1976).

## MATERIALS AND METHODS

The fine structure of the tissues on the back surface of the iris was investigated in a wide variety of available fishes. The following nine species were studied in detail: blue gill, *Lepomis macrochirus* Rafinesque; eel, *Anguilla rostrata* (Lesueur); mummichog, *Fundulus heteroclitus* (Linnaeus); goldfish, *Carassius auratus* (Linnaeus); scup, *Stenotomous chrysops* (Linnaeus); sea horse, *Hippocampus erectus* Perry; three spined stickelback, *Gasterosteus aculeatus* Linnaeus; rainbow smelt, *Osmerus mordax* (Mitchill); rainbow trout, *Salmo gairdneri* Richardson.

The fish were narcotized with Finquel (Ayerst brand of tricaine methane surfonate). If the eye was difficult to enucleate, a window was cut in the cornea and fixative introduced by blunt hypodermic needle to the pupil in a manner to gently flush the back of the iris. The eye was then dissected out of the socket and a circumferential cut made to free the cornea plus part of the sclera and retina, which was then immersed in fixative.

If the eye was large and easily removed, the circumferential cut was made immediately and the front part of the eye immersed then in the fixative.

The fixative was 3% glutaraldehyde together with 1.5% polyoxymethylene (paraformaldehyde) plus 3% sucrose in 0.1 *M* cacodylate buffer adjusted to pH 7.4. Fixation was initiated at room temperature but as soon as the dissections were completed the vials were placed in a refrigerator for six hours. Final trimming was done in cold 0.1 *M* buffer and the tissues left in cold buffer several hours or overnight. Post fixation was done with cold 1% osmium tetroxide in 0.1 *M* cacodylate for 45 minutes. The vials were then brought to room temperature and after several buffer rinses the tissues were stained en bloc with 2% uranyl acetate in 30% acetone. Dehydration was completed in acetone (Baker's Anhydrous 6-A137) and embedment done in Epon 812. Sections were stained with lead citrate.

One of the fixative variations was the use of tannic acid in the first buffer rinse following the primary fixation in an effort to enhance the staining of the tissues (Simionescu and Simionescu, 1976). Results were poor (probably inadequate penetration) except for one fortuitous and unexpected result (see text and Figs. 5 and 10).

## RESULTS

The fine structure of the tissue covering the back of the iris in teleost fishes was examined. The tissue is in the form of a single cell layered nonpigmented epithelium (NPL) backed by a layer of pigmented cells (PL). The two layers extend from the retina to the edge of the pupil and in turn are backed by a layer of connective tissue containing blood vessels (Fig. 1).

The most noticeable and consistent characteristic of the NPL is the baso-lateral interdigitations of the cells. (Note: During ontogeny the NPL is folded inward to cover the PL. Thus, the basal surface of the NPL becomes the free surface in the adult eye). The flattened, leaf-like extensions communicate to the surface of the epithelium and reach to varying depths within a neighboring cell. The cells mutually interdigitate on a one-to-one basis. The occasional occurrence of neighboring light and dark cells demonstrates this clearly (Fig. 4).

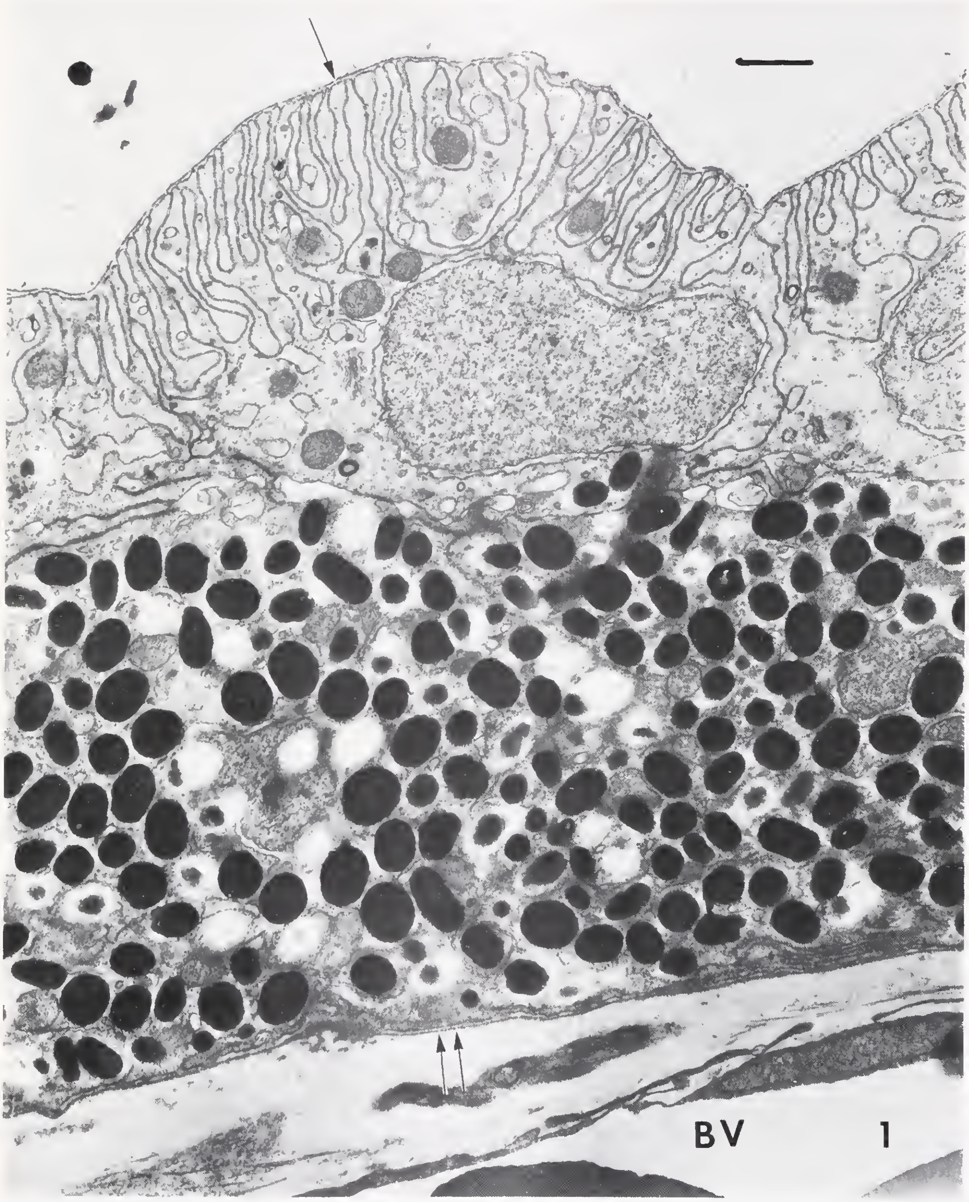

FIGURE 1. *Stickelback.* Low power of both the NPL and PL. The cell in the NPL is covered by an inner limiting membrane (arrow) and shows numerous interdigitating projections from a neighboring cell. The PL cell has a basal lamina (double arrow) and is subtended by a blood vessel (BV). Scale: 1.0 micron.

The inner, vitreal NPL bears an interesting relationship to the subtending PL. Peripherally (*i.e.*, toward the retina) the NPL is devoid of melanin granules. However, as the pupil is approached there is an increasing number of melanin granules to be found in the NPL until at the pupil the two layers are hard to distinguish.

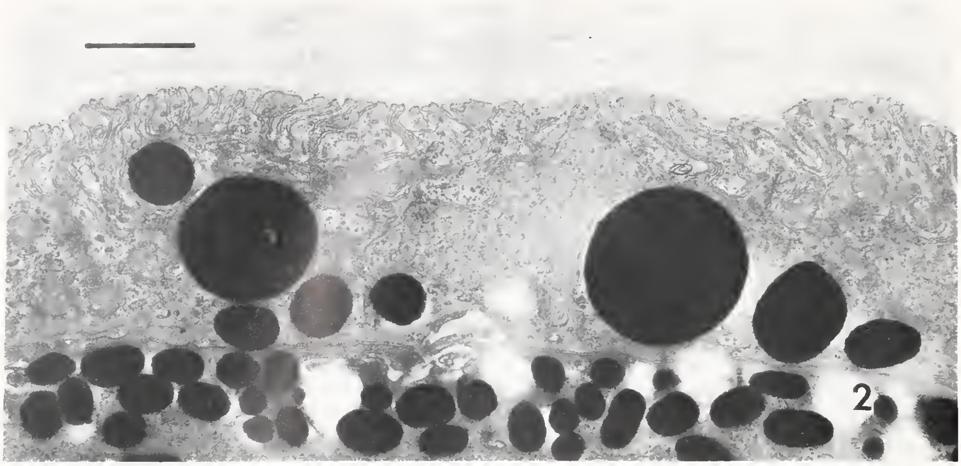

FIGURE 2. *Smelt.* Showing exceptionally large melanin granules in the NPL. Scale: 1.0 micron.

In some instances the granules found in the NPL enlarge enormously (Fig. 2) and in still others they fragment (Fig. 3).

A much less constant characteristic is the nature of the free surface of the NPL. Most of the species examined exhibit a relatively smooth surface. However, goldfish

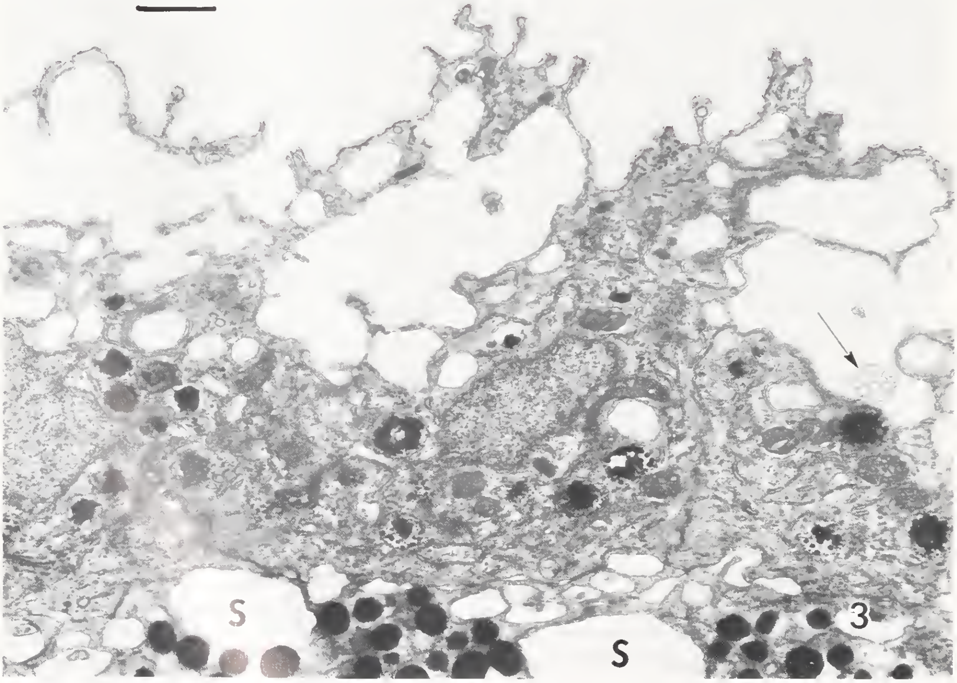

FIGURE 3. *Goldfish.* Several large intercellular spaces (S) are seen between the NPL and PL (ciliary channels?). Multivesiculate body (arrow). Note fragmentation of some of the melanin granules in the NPL. Scale: 1.0 micron.

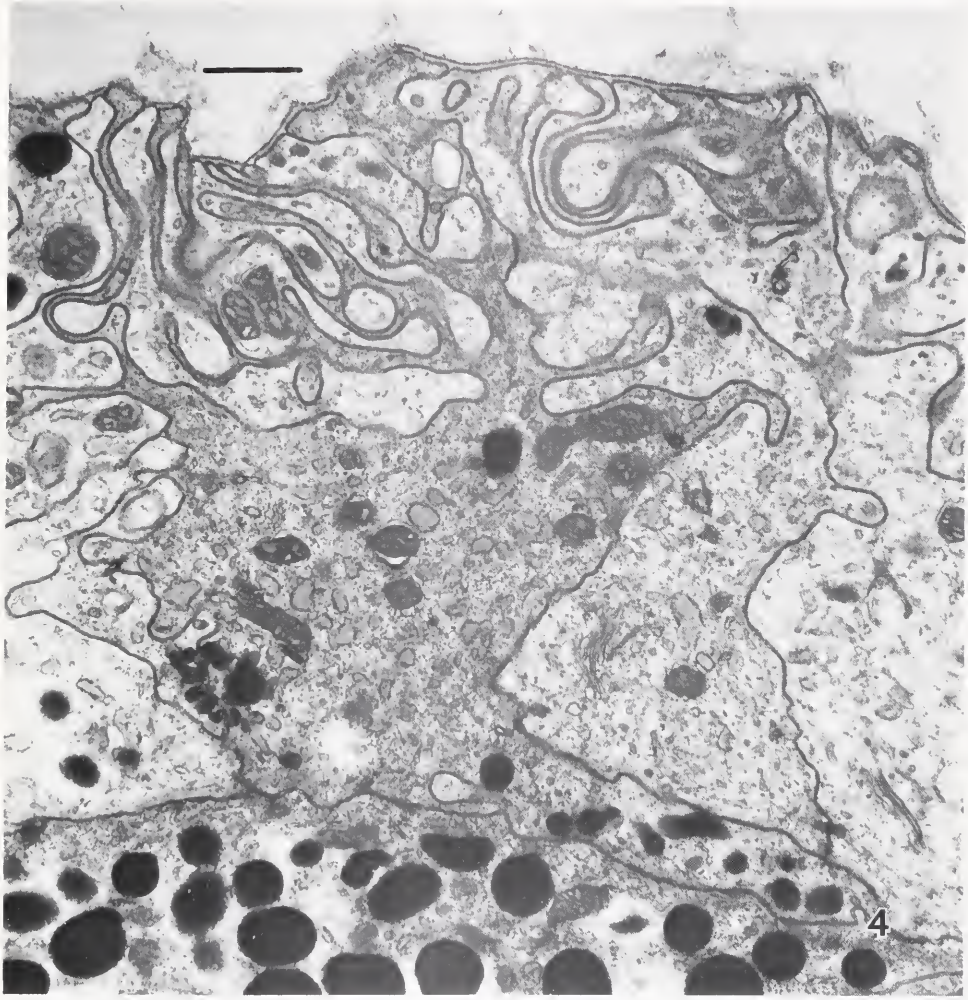

FIGURE 4. *Mummichog.* Low power view of a dark cell with contrasting interdigitations from neighboring light cell(s) of the NPL. Note the one-to-one relationship of the interdigitations. Scale: 1.0 micron.

(Fig. 3), blue gill, and trout have highly irregular, branching folds projecting from the surfaces.

Another characteristic showing a degree of variation is found in the inner limiting membrane on the surface of the epithelium. In most fish it is well developed and strongly adherent (goldfish, Fig. 5 and eel, Fig. 6). In a few it is more fragile and easily lost during the preparative procedures (sea horse, Fig. 7). Due to the happenstance of embryology of the eye, mentioned above, the membrane indeed is a basal lamina.

The mitochondria of all the cells are randomly distributed and showed no preferential orientation to the interdigitated projections. However, they are included sometimes within the more blunt ones (Fig. 1).

Well-developed Golgi apparati and associated membranous structures are located in the scleral end of the NPL. Secretory granules may be seen in the same region (Fig. 8).

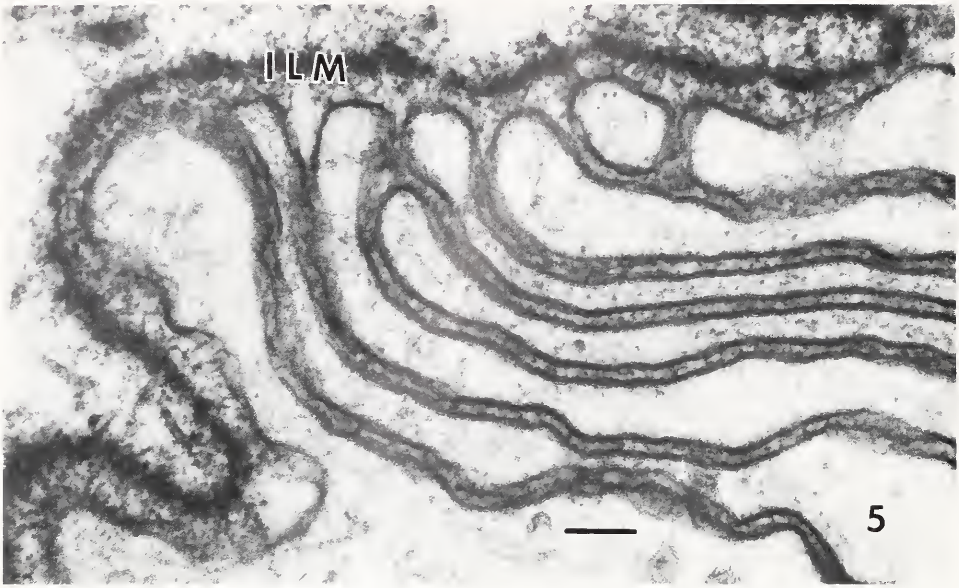

FIGURE 5. *Goldfish.* Surface of the NPL. The inner limiting membrane (ILM) is well developed and adherent. Penetration of tannic acid mordant delineates the intercellular spaces of the interdigitations and the granular material within the spaces. Note there are no cellular junctions. Scale: 0.1 micron.

Although not preserved in all preparations, coated vesicles are seen frequently (Fig. 9). Usually, they are found associated with the free surfaces of the cells.

The NPL has both rough endoplasmic reticulum (usually in the Golgi area) and smooth endoplasmic reticulum throughout the cell.

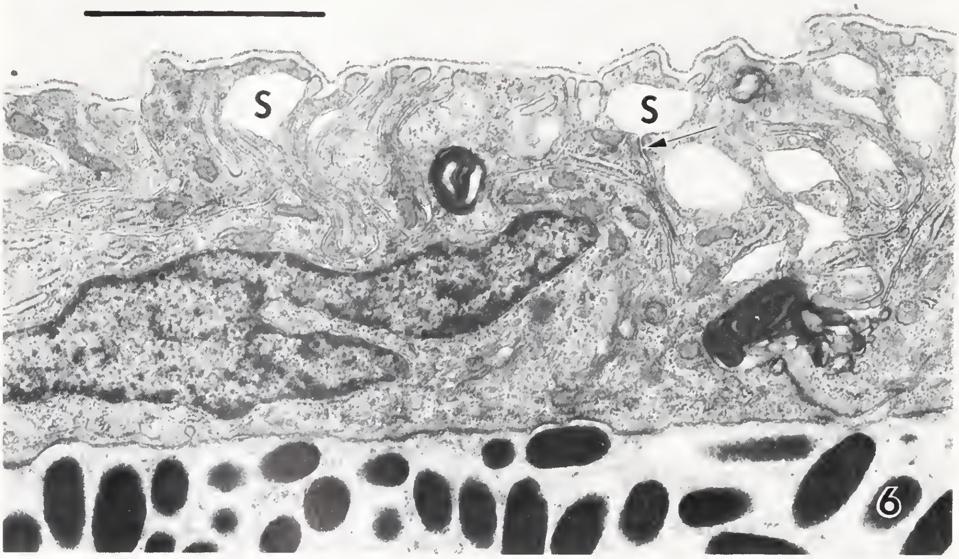

FIGURE 6. *Eel.* Inner limiting membrane well developed. Intercellular spaces are seen (S). A macula type junction or desmosome (arrow) common to the cell body plasma membranes (not to the interdigitations) is seen adjacent to one of the spaces. Scale: 0.1 micron.

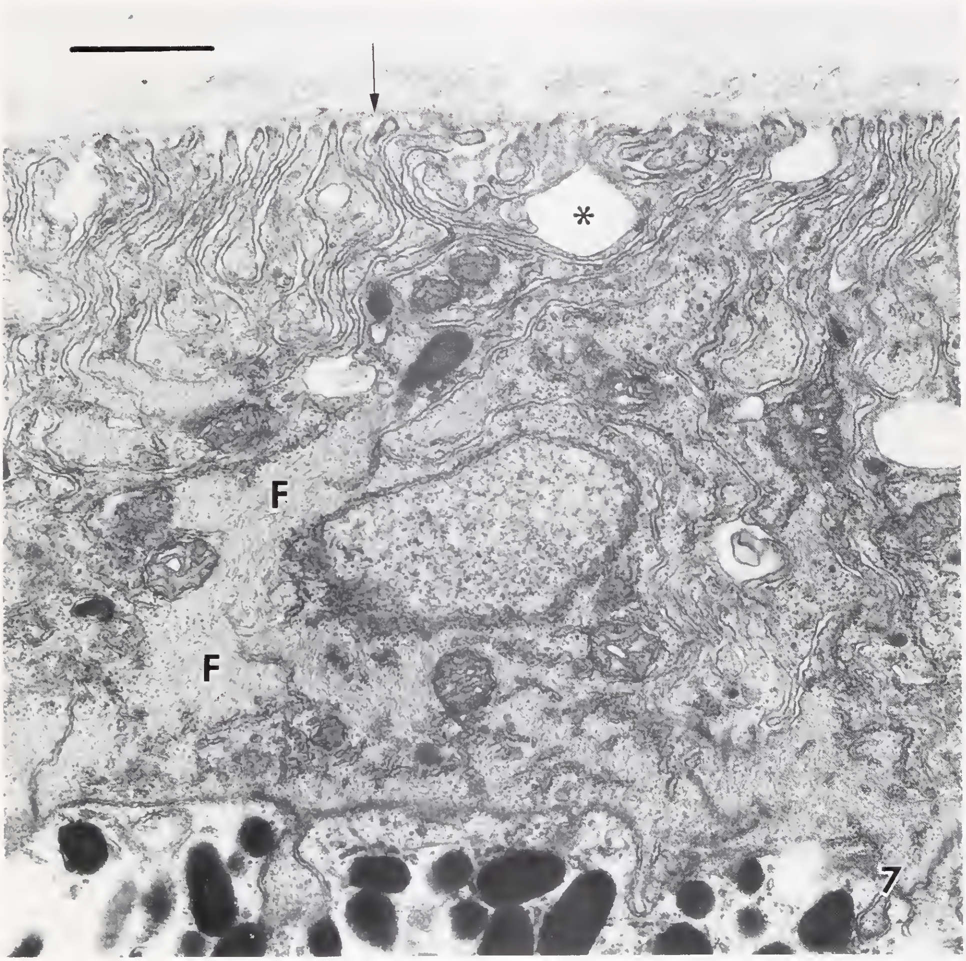

FIGURE 7. *Sea Horse.* A weakly organized inner limiting membrane (arrow) rests on the interdigitations and a few fibers common to the vitreous humor are above the membrane. A high concentration of filaments (F) is present within the NPL cell. A well-developed intercellular space (asterisk) is seen near the surface. Scale: 1.0 micron.

Microtubules are seen occasionally in the cell surface areas, but much more predominant are clusters of small fibrils (Fig. 10). In one fish, sea horse, they occupy a good share of the cell cytoplasm (Fig. 7). They are of the order of intermediate or 10 nm filaments. That is, they are "intermediate" to microtubules at 24 nm and microfilaments at 5–7 nm.

The space between the interdigitating plasma membranes of the adjoining cells of the NPL is open to the free surface. Due to a fortuitous usage of tannic acid technique, in one instance the plasma membranes are not only selectively stained but particulate material is seen in the intercellular space (Figs. 5 and 10). The same type of particles seen beneath the inner limiting membrane is found also between the cells (Fig. 5). Desmosomes are found at random intervals between the plasma membranes of the cell bodies proper, but are seen rarely between the membranes of the complementary interdigitations.

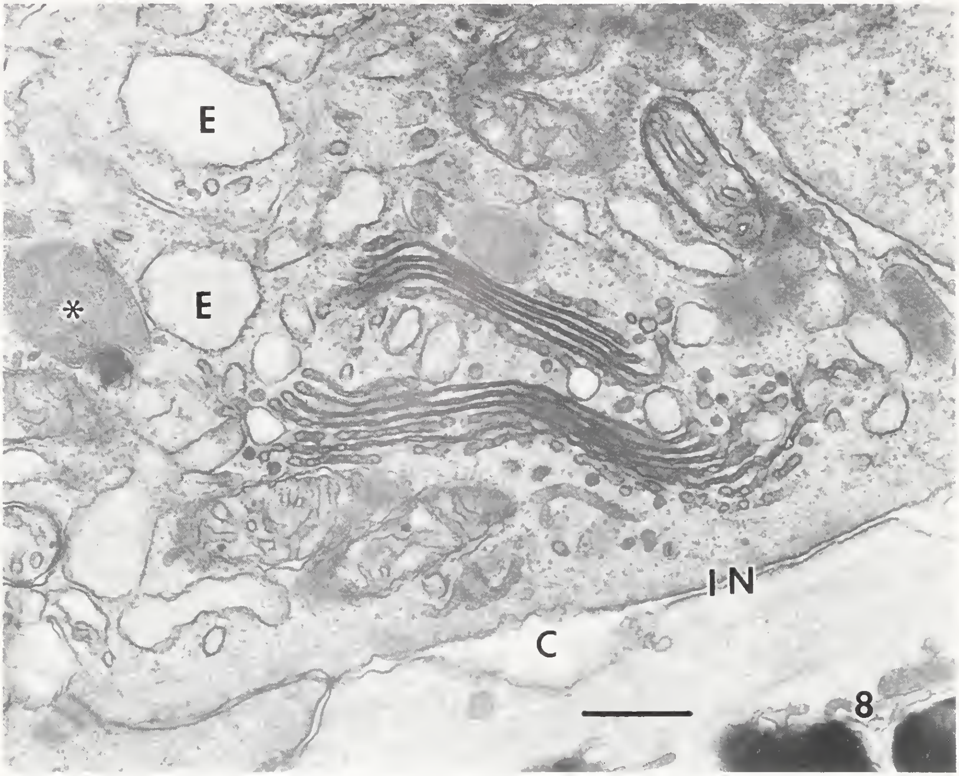

FIGURE 8. *Scup.* Typical Golgi apparatus within the NPL and adjacent to the PL interface (IN). Expanded endoplasmic reticulum (E) with granular material. Secretory granule (asterisk). Ciliary channel (C). Scale: 1.0 micron.

The size and number of intercellular spaces in the NPL varies from species to species. They may be almost nonexistent, as in stickelback (Fig. 1), smelt, scup, mummichog; small, eel (Fig. 6) and sea horse (Fig. 7); large, goldfish (Fig. 3) and blue gill; or very large, trout. In some cases, spaces are seen also between the NPL and PL (Fig. 8). The spaces frequently contain fine granular material and, at times, membranous, multivesiculate material.

The PL shows little activity compared to that seen in mammalian species. The cells are filled with melanin granules and have only a few structures such as mitochondria, Golgi apparati, endoplasmic reticulum, *etc.* Occasionally, a mild degree of interdigitation occurs between the end of the cells facing the vascular vessels. There also are occasional intercellular spaces filled with granular material similar to that seen in the spaces of the NPL.

## DISCUSSION

My observations on the fine structure of the goldfish NPL are not in complete agreement with Zadunaisky's description of the same species. His "microvilli" are in reality tortuous folds or outpocketings of the cell surface. Also he did not note the cellular interdigitations to be found in the NPL of the fish that he describes (goldfish). The interdigitations may not be as numerous or complex as in other fish, but they are present.

The interdigitation of the neighboring cells is the most consistent feature common to all the fish studied. The dimensions of the interdigitations vary somewhat from species to species but that is not an artifact (*i.e.*, exactly the same fixative procedures were used throughout). The differences may be due to slight differences in the tonicity and/or ionic balance in the respective aqueous humors, factors not known at present.

Also, noteworthy is the fact that a plicated or ruffled surface of the NPL is found only in three fresh water forms (goldfish, blue gill and trout). The surfaces are smooth in the six sea water species (and in a number of other sea water fishes not described here). Although suggestive of a true difference beween fresh water and sea water fish, a greater number of fresh water fish would need to be examined to determine the validity of such an indication.

The fine structure of the epithelium on the back of the teleost iris bears a close and striking resemblance to the ciliary epithelium of the mammals.

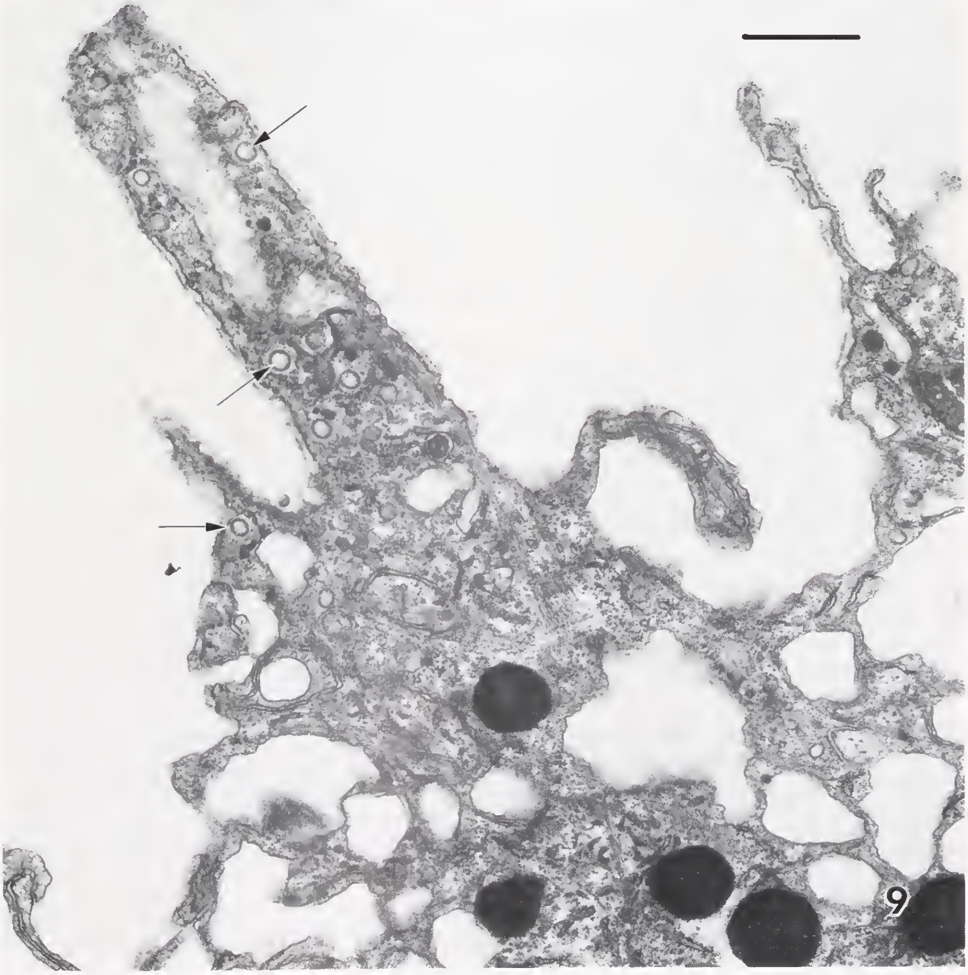

FIGURE 9. *Goldfish*. Tip of one of the surface ruffles showing coated vesicles (arrow). Scale: 1.0 micron.

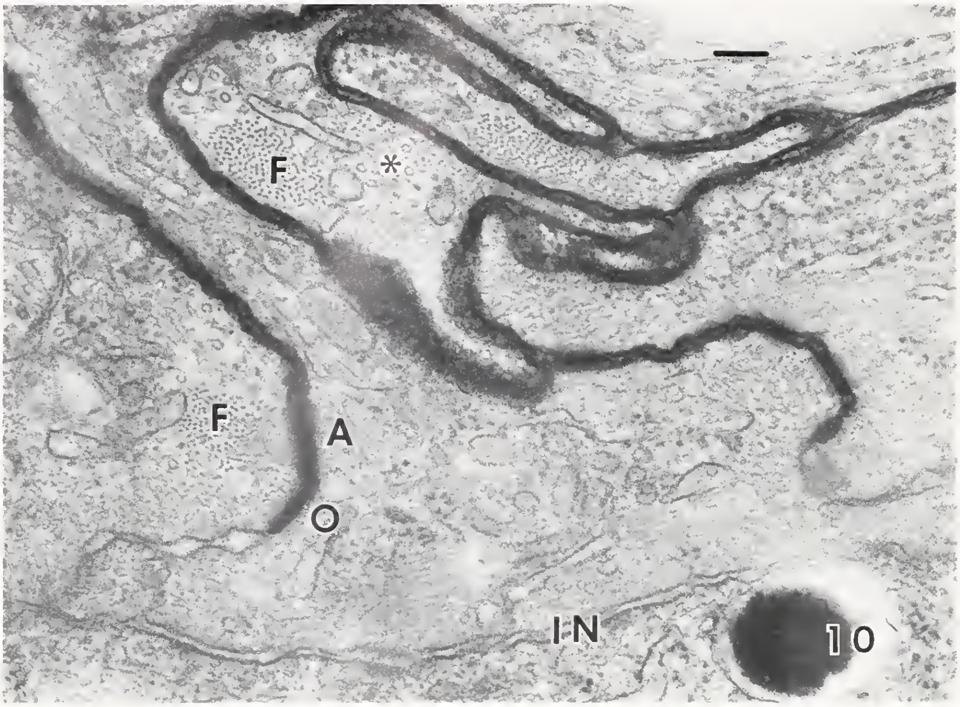

FIGURE 10. *Goldfish*. NPL at its interface (IN) with the PL. From same tissue block as in Figure 5 but at a lower power. Note that the penetration of tannic acid into the intercellular space is limited by the zonula occludente (O). Cross section of intermediate filaments (F). A few microtubules (asterisk). Zonula adherente (A). Scale: 0.1 micron.

Highly noteworthy is the existence of cellular interdigitations in the NPL of fish. These are of a type and orientation similar to those seen in mammals (references listed in the Introduction). The interdigitation in teleosts is on a one-to-one basis as demonstrated by the fortuitous association of light and dark cells. Tormey (1963, 1964) and Kaye and Pappas (1965) made the same type of observations in the rabbit ciliary epithelium.

Equally significant, the barrier of zonulae occludentes and associated zonulae adherentes found at the apex of the NPL of mammals (Bairati and Orzalesi, 1966; Shabo and Maxwell, 1972; Uusitalo *et al.*, 1973; Raviola, 1974; Okisaka, 1976b) is found also in fish. Though none of the usual tracers were used in the present investigation, the happenstance of limited tannic acid penetration between plasma membranes validates this interpretation.

The NPL shows all the fine structure usually seen in secretory cells. There is a plentiful supply of membranous organelles such as Golgi apparati, endoplasmic reticulum (rough and smooth), mitochondria, and granules filled with particulate material.

The PL is packed with the melanin granules and shows almost none of the morphology usually associated with metabolic or secretory activity. The basal-lateral surfaces of the cells show a mild degree of interdigitation but in no way approach the complexity seen, for example, in the monkey (Okisaka, 1976a).

One of the prime "road blocks" found in the current literature is the commonly held belief that the zonulae occludentes junctions in the NPL represent an inviolate blood-aqueous barrier. The work of Raviola (1974) gives most excellent support to this idea. Nevertheless, it should be kept in mind that living cells are dynamic systems and they could well eliminate and reform junctions as they do other organelles. It is of puzzling significance that the intercellular spaces between the NPL and PL (and frequently within the PL) have the same appearing content (multivesiculate or granular) as the intercellular spaces in the NPL. The finely granular material is seen consistently enough to suggest that it is a normally occurring material. However, the multivesiculate type clusters, also sometimes seen in the spaces, occur randomly enough that they could be artifacts.

If, as repeatedly stated in the literature, there is indeed an inviolate blood-aqueous barrier in the distal borders of the NPL by reason of zonulae occuludentes, then attention must be turned to the basal interdigitations where only local, maculae type junctions occur infrequently. Here interpretation of function, though indirect, can be made more plausible as explained below.

Zadunaisky (1972) has shown that the aqueous humor in two teleost fishes (goldfish and the marine sargus) is hypotonic to the blood plasma, contrary to the situation in mammals and amphibia. His experimental physiological procedures indicated that sodium and potassium are preferentially absorbed to effect the lowered tonicity. Later, at the fine structure level he found a histochemical localization of ATPase on the free surface of the NPL (Zadunaisky, 1973). He interprets this as a possible site for the metabolic pump that could account for the absorption of electrolytes during the formation of the *hypotonic* aqueous of the fish eye. It is of related significance that Kaye and Pappas (1965) found ATPase on the free surfaces and interdigitations of the equivalent tissue in the rabbit. They, however, interpret the presence of the enzyme as facilitating the secretion of electrolytes in the formation of the *hypertonic* aqueous of the rabbit eye.

There already exists an excellent example of a one-to-one interdigitation in a tissue whose function is specifically osmoregulatory. The marine blue crab, *Callinectes sapidus*, invades very dilute marsh areas in the warmer months in search of food and are found in waters with as low as 0.5‰ total salinity. Salt is then absorbed through an epithelium that lines a part of the vascular space of the gills. Physiological proof of salt absorption by crab gills was provided by Nagel (1934) and Koch *et al* (1954). The fine structure of the single cell layered epithelium has been studied by Copeland and Fitzjarrell (1968). The cells laterally interdigitate quite deeply on a one-to-one basis (see Fig. 7, page 8, Copeland and Fitzjarrell, 1968).

The striking similarity between the crab gill tissue, which is specifically devoted to salt transport, and the NPL of fish and mammals is remarkable. This morphological coincidence plus the physiological determinations made by Zadunaisky (1963) suggest that the NPL has an osmoregulatory function related to the ultimate producton of aqueous humor.

Thus, at the morphological level, two functions can be suggested for the combined NPL and PL of the fish iris. One, the presence of similar intercellular granular material in both the layers suggest a secretion of aqueous humor precursor by way of the spaces between the cell bodies. Two, the plicating interdigitations of the NPL may refine the aqueous humor by means of absorbing specific electrolytes.

## ACKNOWLEDGMENT

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# FUNCTION OF CHEMORECEPTOR ORGANS IN SPATIAL ORIENTATION OF THE LOBSTER, *HOMARUS AMERICANUS*: DIFFERENCES AND OVERLAP

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## ABSTRACT

Three of the lobster's main chemoreceptor organs, the lateral and medial antennules (representing smell) and the dactylus-propodus segments of the walking legs (representing taste), are physiologically quite similar. We examined their role in spatial orientation in a food-odor stimulus field.

Control animals almost always oriented correctly and immediately to an odor plume. Lobsters with unilateral ablations of lateral antennules lost this ability, but did not show preferential turning toward the intact side. Unilateral medial antennule ablation did not affect orientation. Removal of all aesthetasc hairs from one lateral antennule caused loss of orientation ability less severe than unilateral ablation of the entire lateral antennule. Lobsters with unilaterally ablated lateral antennules and blocked walking leg receptors turned preferentially toward the side of the intact antennule.

Thus, it appears that intact lobsters orient in odor space by tropotaxis principally using aesthetasc receptor input. The first two pairs of walking legs and non-aesthetasc receptors on the lateral antennule have additional roles in spatial chemical orientation. The medial flagellum does not contribute to orientation. Since loss of appendages is relatively common in lobsters, this partial overlap of organ function may serve the animal well in nature.

## INTRODUCTION

In the American lobster, searching for food may be elicited and maintained by chemical cues alone. In order to search efficiently the lobster must be able to identify a chemical cue and extract directional information from a chemical stimulus field. Several bilateral chemoreceptor organs located on different appendages must be considered as possible mediators of distance orientation.

The biramous antennules are usually considered the distance chemoreceptors in decapod crustaceans (Maynard and Dingle, 1963; Hazlett, 1971). In particular, the aesthetasc hairs of their lateral flagellum are implicated as being chemoreceptors by morphological (Laverack, 1964; Laverack and Ardill, 1965; Ghiradella *et al.*, 1968), electrophysiological (Ache, 1972; Shepheard, 1974), and behavioral studies (McLeese, 1970, 1973, 1974; Snow, 1973; Reeder and Ache, 1980). Walking leg and maxilliped chemoreceptors have been described respectively as "outer" and "inner" contact chemoreceptors (Luther, 1930). On the walking legs the regions of greatest receptor density and specialization are the dactylus and propodus (Derby and Atema, 1982a); the walking leg chemoreceptors are often incorrectly called

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dactyl receptors. Specific chemoreceptor sensilla on dactylus and propodus have been identified in crayfish (Hatt and Bauer, 1980), and in the lobsters *H. gammarus* (Shelton and Laverack, 1968, 1970) and *H. americanus* (Derby, 1982). Roles of these different chemoreceptor organs in feeding behavior of *H. americanus* were described by Derby and Atema (1982b). The external appearance of chemoreceptor sensilla may have been shaped by their micro-environments; for example, antennular chemoreceptors remain in the water column, while leg chemoreceptors are subjected to abrasion when the lobster is walking or probing in the substrate (Atema, 1980). Despite morphological differences of sensilla, primary receptor cells of antennules and walking legs may be quite similar in response spectrum and threshold. Although earlier studies found leg chemoreceptors to have higher thresholds than antennular receptors (Case and Gwilliam, 1961; Ache, 1972; Shepheard, 1974; Fuzessery and Childress, 1975; Fuzessery *et al.*, 1978), recent work has shown that both leg (Derby and Atema, 1982a) and antennular receptors (Thompson and Ache, 1980) can have thresholds lower than previously known. Based on their physiology both antennules and legs could be efficient distance chemoreceptors. Studies which correlate this physiological and morphological information with behavioral function are scarce, and the labels "contact" and "distance" chemoreceptor are based on casual observation only. However, based on neuroanatomical and behavioral criteria, the crustacean antennules can be called smell organs and the legs and maxillipeds taste organs in analogy with vertebrates and in homology with insects (Atema, 1980).

An increase in the rate of antennule flicking, *i.e.,* the periodic depression of the lateral flagellum of the antennule, is generally one of the first observable changes in behavior after chemical stimulation. This behavior has been used to determine chemical detection thresholds (Pearson and Olla, 1977). Thresholds are effectively lowered by flicking (Schmitt and Ache, 1979). Flicking—functionally similar to sniffing by terrestrial vertebrates—may well compensate for the haphazard spatial and temporal character of a chemical stimulus field. This and both physiological (Fuzessery, 1978) and behavioral evidence (McLeese, 1973; Reeder and Ache, 1980) strongly argue for the importance of the lateral flagellum in spatial orientation. After bilateral ablation of the aesthetasc-bearing flagella, spiny lobsters did not search in response to food odor, whereas ablation of the medial flagella did not interfere with searching behavior (Reeder and Ache, 1980). This animal uses both tropotactic and klinotactic components in orientation to food odors.

This study shows that *H. americanus* appears to orient to odors principally by means of the aesthetasc receptor input, that other chemoreceptors on the lateral flagellum of the antennules may contribute somewhat to orientation, and that the walking leg chemoreceptors function in orientation when aesthetasc input is impaired.

## MATERIALS AND METHODS

### *Materials and apparatus*

Lobsters used in this study (carapace length 60–82 mm) were captured by local fishermen in the waters off Woods Hole, Massachusetts. They were maintained in holding pens in running seawater for at least two weeks prior to placement in testing tanks.

All tests were done in three identical 675-liter fiberglass aquaria measuring 1.25 m long × 0.9 m wide × 0.6 m deep with glass fronts. Each tank was outfitted with a biologically conditioned sub-gravel filter and filled with either natural or artificial seawater. Illumination was provided by a 40 W bulb suspended 1 m above the water surface. The light cycle approximated natural sunrise and sunset for that particular time of year. The water temperature varied from 18 to 22°C. A single animal was kept in one tank for the entire length of an experiment. Animals were allowed a minimum acclimation period of two days before any tests were run. The lobsters were fed daily on a diet of cod muscle (*Gadus callarias*), but never to satiation; they were never fed at the odor source locations used in tests.

Each tank was fitted with a double symmetrical recirculating seawater system as described by Atema and Gagosian (1973). The airlift water intakes were above the lobster's shelter, in the rear center of the tank. Each intake delivered an irregular flow of about 25 ml/sec. Funnel interruptions in both sides of this system allowed introduction of a chemical stimulus without appreciable (2–3%) concurrent novel mechanical stimulus. A 2–4 min time delay further separated chemical stimulus arrival from possible contamination with a mechanical stimulus. Water flowed down the stem of the funnel to a right angle glass elbow facing perpendicular to the side of the tank and located approximately 4 cm from the bottom of the tank. This outflow was covered with rocks and located inside the center hole of a three-hole cinderblock to protect the all-glass system from the test animals (Fig. 1).

The stimulus used was an extract of homogenized and filtered cod muscle at a concentration of 5 g wet weight/l water. The stimulus side was semi-randomly switched between left and right; however the total number of trials was divided equally between the two sides. The stimulus was presented by pouring a 2-ml dose into the funnel over a 3-sec interval. Dye studies showed that traces of the stimulus remained in the funnel system for up to 30 sec, and that the dye pulse was visible at the shelter between 2 and 4 minutes after introduction. This variation in arrival times was due to variation in water currents between tanks; variation among tests within each tank was 15–30 sec ($N = 4$). The stimulus pulse had been diluted by a factor of $10^3$ to $10^4$ upon reaching the lobster in its shelter as measured by colorimetric analysis with methylene blue dye. The stimulus front moving from these outflows approached perpendicular to the length of the lobster in the shelter; the odor space appeared typically haphazard with whirls, lines, and pockets of various concentrations of dye.

### *Testing methods*

Observations were made in a darkened room with the observer seated in front of the tank. All observations were made during the day. Lobsters are naturally nocturnal. Light keeps them in shelter, but when low enough it does not prevent them from searching when a chemical food stimulus is presented. All trials were run as described below.

Once the lobster was quiet and in its shelter, a single stimulus dose was introduced into the tank via the funnel system. The following three measurements on orientation were made: latency to alert, initial direction choice, and search path. The time period from stimulus introduction to the lobster's first behavioral response, "alert," was recorded. Among various possibilities we chose to define "alert" as distinct waving and pointing of the second (large) antennae and sudden body movements. If no alert response was observed within 8 minutes, an animal was recorded as giving "no response." Following alert and upon exit from the shelter, a searching lobster made an initial direction choice, either to the left or right of an imaginary line down the center of the tank. References to handedness always refer to the perspective of a lobster in its shelter, not to the observer. This initial direction

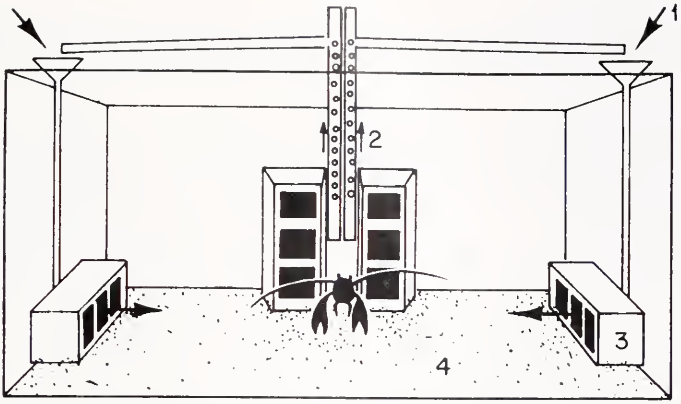

FIGURE 1. Diagram of test aquarium: (1) chemical stimulus, (2) air lift, (3) brick, (4) gravel substrate.

choice was scored as either "+" or "−" with respect to the stimulus side or "no response" if searching was not elicited. A sketch was made of the path taken by the test animal while it searched for the stimulus source. A test was considered completed when either an animal located the stimulus source or 10 minutes had elapsed since the introduction of the stimulus. The search path lengths were subsequently measured with a planimeter and converted back to actual distances walked by the test animal. Although preliminary tests showed that a lobster's performance was not affected by tests as close together as 1.5 hours, 3 to 12 hours were generally allowed between tests. Two to four tests were run per animal per day.

*Treatment groups*

The experiments were organized into four treatment groups of six lobsters each. In all groups, fifteen trials were run per animal to establish a baseline. The same number of trials was run on each animal after each phase of treatment. The treatments were: 1) The right lateral antennular flagellum was ablated, then the right medial flagellum was subsequently ablated. 2) The sequence of treatment 1 was reversed: ablation of the right medial flagellum was followed by right lateral antennular ablation. 3) The aesthetasc hairs of the right lateral flagellum were shaved off with a scalpel while the lobster was cold-anaesthetized on its back in a tray of ice. Once the experiment was completed, the shaved flagella were removed and prepared for scanning electron microscopy. 4) The dactylopodite and propodite of all four pairs of walking legs as well as the seizer claw were coated with the cyanoacrylate adhesive KrazyGlue® which formed a waterproof acrylic "glove" around the leg when dry. The use of cyanoacrylates does not result in non-specific behavioral changes in *H. americanus* (Derby and Atema, 1982b; C. Derby, unpublished data). These animals underwent subsequent ablation of the right lateral antennular flagellum. The right side of all animals was consistently used for ablation since there is no evidence for dominance of left or right in processing chemosensory input. Eighteen-hour recovery periods were allowed following all treatments for recovery from the effects of ablation and handling.

Friedman's analysis of variance by ranks ($X_r^2$) and multiple comparisons for ranked data were performed using the sums of each fifteen-trial group (Zar, 1974).

For presentation purposes, means and standard errors were calculated for each treatment group. Significance was accepted at the 0.05 level.

## RESULTS

Normal lobsters initially responded to cod muscle extract by waving their antennae, increasing the rate of antennule flicking, and changing body stance and claw posture from resting to walking. Following this alert response and a short wait in the shelter, the lobster left the shelter in search of the odor source. Initial direction choice was almost always correct (347 times out of 360 tests). Searching consisted of walking a generally straight path to the odor source while doing much antenna waving, antennule flicking, fanning the exopodites of the maxillipeds, pleopod beating, and occasional wiping of antennules by the third maxillipeds. Within one body length of the odor source, the animals often probed the substrate with walking legs and maxillipeds. Upon reaching the odor source, normal animals would attempt to reach the outflow by inserting their first and second walking legs into the cinderblock where the odor outflow was hidden. They often persisted in this directed search behavior for several minutes.

None of the treatments affected either the lobster's use of antennae and walking legs in searching or their probing with walking legs and moving of maxillipeds. Also, none of the treatments affected alert latency (Tables I and II). However, in

TABLE I

*Effects of chemoreceptor appendage ablations on three behavioral parameters.*

| | Alert latency (sec) $\bar{X} \pm$ SEM | Correct direction choice (15 trials) | Search path length (cm) |
|---|---|---|---|
| Group 1 | | | |
| a) Untreated | 106.7 ± 9.5 | 14.3 ± 0.3 | 159.3 ± 30.0 |
| b) Right lateral ablation | 102.2 ± 9.8 | 7.8 ± 0.3 | 250.5 ± 34.3 |
| c) Subsequent right medial ablation | 91.5 ± 14.9 | 8.0 ± 0.4 | 218.5 ± 33.5 |
| Statistic and significance | $X_r^2 = 5.33$ NS | $X_r^2 = 9.08$ $P < 0.02$ | $X_r^2 = 10.3$ $P < 0.01$ |
| Group 2 | | | |
| a) Untreated | 153.0 ± 16.0 | 14.2 ± 0.3 | 105.3 ± 4.7 |
| b) Right medial ablation | 164.2 ± 17.9 | 14.8 ± 0.2 | 103.5 ± 3.7 |
| c) Subsequent right lateral ablation | 171.2 ± 21.2 | 8.8 ± 0.5 | 209.2 ± 14.8 |
| Statistic and significance | $X_r^2 = 2.3$ NS | $X_r^2 = 9.34$ $P < 0.01$ | $X_r^2 = 9.33$ $P < 0.01$ |
| Group 3 | | | |
| a) Untreated | 171.2 ± 14.4 | 14.7 ± 0.2 | 111.7 ± 10.0 |
| b) Right aesthetascs shaved | 195.7 ± 20.0 | 9.8 ± 0.8 | 236.8 ± 35.5 |
| Statistic and significance | $X_r^2 = 2.6$ NS | $X_r^2 = 6$ $P < 0.05$ | $X_r^2 = 6$ $P < 0.05$ |
| Group 4 | | | |
| a) Untreated | 167.3 ± 20.7 | 14.7 ± 0.2 | 98.0 ± 3.3 |
| b) Glue-covered legs | 172.8 ± 24.0 | 14.8 ± 0.2 | 110.2 ± 4.9 |
| c) Subsequent right lateral ablation | 180.8 ± 22.2 | 8.3 ± 1.0 | 271.3 ± 12.9 |
| Statistic and significance | $X_r^2 = 5.33$ NS | $X_r^2 = 9.0$ $P < 0.02$ | $X_r^2 = 9.33$ $P < 0.01$ |

NS = not significant.

TABLE II

*Behavioral changes in food odor orientation after chemoreceptor appendage ablations.*

| Group | Ablations | Change in alert latency | Correct direction choice (%) | | Change in search path length (%) | | Circus movement |
|---|---|---|---|---|---|---|---|
| 1a | None | — | 94 | | — | | no |
| 1b | Lateral (L) | no | 52° | | +71** | | no |
| 1c | L + M | no | 53° | | +44** | | no |
| 2b | Medial (M) | no | 99 | * | +1 | | no |
| 2c | M + L | no | 59** | | +101** | ** | no |
| 3b | Aesthetasc | no | 66* | | +108* | | no |
| 4b | Legs Coated (C) | no | 99 | | +14 | | no |
| 4c | C + L | no | 56° | | +177** | | yes |

Group numbers and treatments are the same as in Table I.
Statistical significance: $*P < 0.05$, $°P < 0.02$, $**P < 0.01$ (Mann-Whitney U-test).

all treatment groups, both initial direction choice and subsequent search path length were significantly altered by ablation of the right lateral flagellum or by removal of aesthetasc hairs regardless of the presence (groups 1b, 3b, and 4c) or absence (group 2c) of the medial flagellum. In contrast, glue-coating all walking legs (group 4b) did not change initial direction choice nor search path length (Tables I and II), and ablation of the medial flagellum before (group 2b) or after (group 1c) ablation of the lateral flagellum had no effect on any of the measured behavioral parameters.

SEM showed that the aesthetasc hairs of animals in treatment group 3 were indeed removed. Even in the least effective shaving (Fig. 2), only the bases of some sensilla remained. The significant decrease in the number of correct initial direction choices and the concomitant increase in search path length caused by aesthetasc shaving was not as great as the decrease caused by lateral flagellum removal; the difference is significant in itself (Table II).

In contrast to lobsters in other treatments, only leg-coated lobsters with unilateral ablation of the right lateral flagellum (group 4c) made a significantly higher number of initial direction choices to the left (*i.e.* intact) side ($X_r^2 = 9.33$; $P < 0.01$) regardless of stimulus direction. In many of the trials these lobsters made complete left-turning circles while searching (circus movements, Table II). Besides becoming more erratic these animals searched more slowly, and the increase in length of their search paths was significantly greater ($P < 0.01$) than that of lobsters with only lateral flagellum ablations (Table II). Outside the experimental observation regime, during feeding such lobsters had difficulties locating their daily food.

## DISCUSSION

Since none of the experimental manipulations altered alert latency, and assuming that latency across animals and experiments is correlated with detection threshold, we conclude that the lobsters' threshold for odor detection and identification at this stimulus concentration was not affected by unilateral antennule ablations and/or glue-covered legs. The results of experiments 1 and 2 demonstrate the importance of lateral flagellar chemoreceptors for the extraction of directional information from a chemical stimulus field. This is reflected in the dramatic shift in correct initial direction choice from nearly 100% to roughly random following

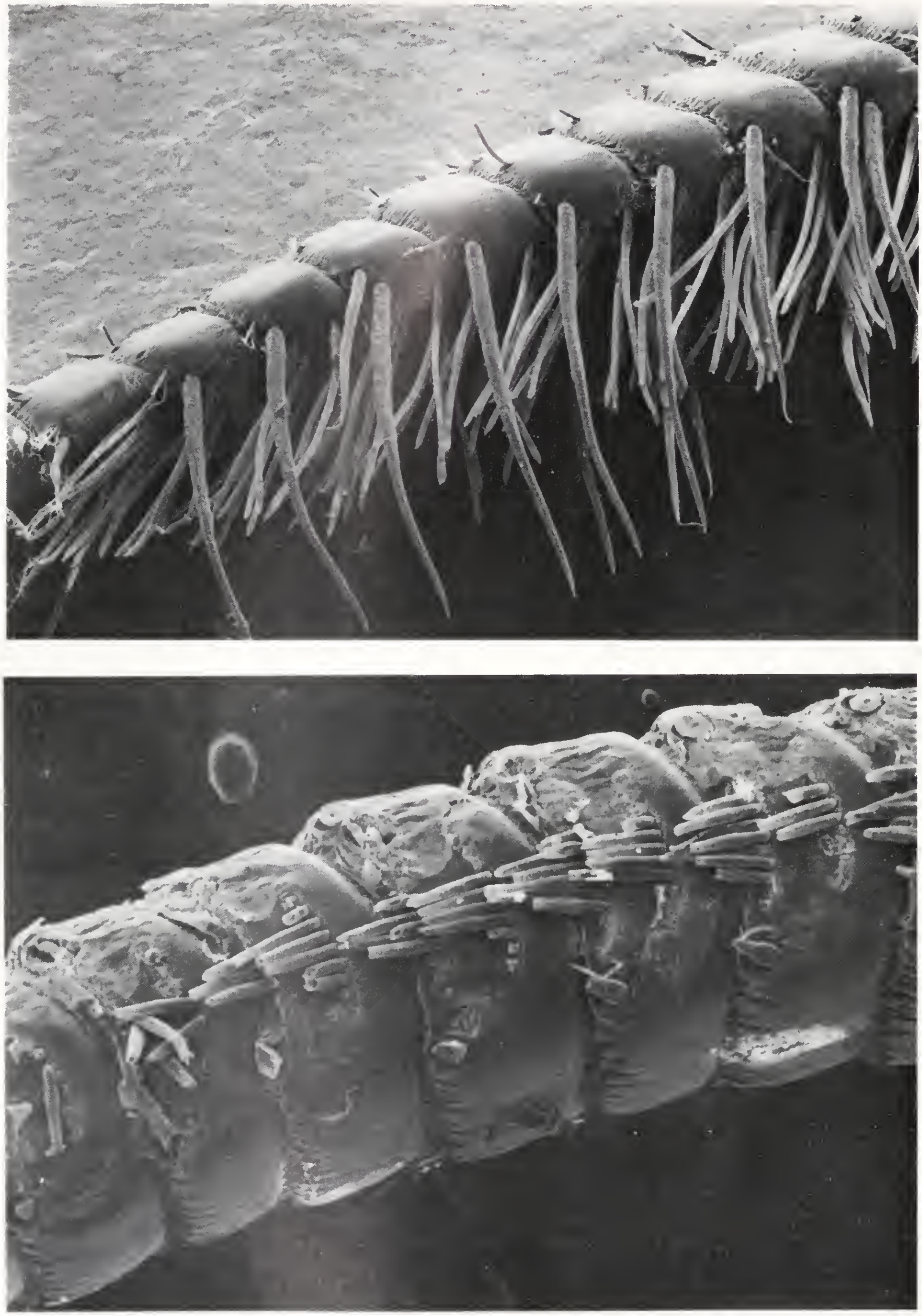

FIGURE 2. SEM of portion of lateral flagellum of antennule. Top: (A) Normal rows of 8–12 aesthetasc hairs, 2 rows per segment, flanked by much larger guard hairs. Bottom: (B) Aesthetasc and guard hairs shaved off at base; in this worst example several remaining aesthetasc stumps contain only proximal segments of receptor dendrites.

unilateral ablation of the lateral flagellum. The same effects are seen when search path length is used as a measurement of orientation efficiency. Ablation of the ipsilateral medial flagellum either prior to or following lateral ablation had no effect on searching behavior, indicating that the input from medial flagellum receptors was not necessary for efficient orientation to odors.

Removal of aesthetasc sensilla alone was sufficient to cause significant changes in orientation ability, but it did not affect initial direction choice as much as entire lateral flagellum ablation (Table II). Therefore, input from other, unidentified lateral flagellum chemoreceptors may aid in orientation. There is both physiological (Fuzessery, 1978) and morphological evidence (Laverack, 1964; Derby, 1982; Gleeson, 1982) for the existence of non-aesthetasc receptors. Since the lateral flagellum is adapted to temporal and spatial sampling of odor space through a combination of its morphology and flicking behavior (Schmitt and Ache, 1979), one could hypothesize that the entire chemosensory input from this flicking appendage is useful for spatial orientation. Yet, the aesthetasc sensilla probably carry the bulk of the information if only by the sheer number of their afferent neurons, about 400 per sensillum (Oleszko-Szuts and Atema, 1977). The behavioral experiments reported here support this notion and extend the results of antennule impairments obtained for spiny lobsters (Reeder and Ache, 1980) by identifying the aesthetasc input as the main but not the exclusive source of directional information. The alternate explanation that the remaining aesthetasc hair bases retain partial function cannot be rejected, but is in our opinion less likely, based on electron microscopic observation of receptor morphology. The base of the aesthetasc hair is made of thick, lamellated cuticle, lined inside with supporting cells. At the level of the transition from the base to the distal portion, inside the hair are the ciliary junctions of the receptor cells (Oleszko-Szuts and Atema, 1977). Thus all of the receptor cell distal segments were removed in the incomplete shavings. Interactions of stimulus and receptor molecule presumably occurs in the ciliary distal segments.

If lobsters are using tropotaxis to make their initial direction choice, unilaterally ablated animals would be expected to show preferential turning toward the intact side regardless of the direction of the stimulus (Fraenkel and Gunn, 1961), resulting in circus movements, *i.e.* turning circles in the direction of the intact side when stimulated. Such circus movements in chemical gradients were described for spiny lobsters with unilateral antennule ablation (Reeder and Ache, 1980) but they were not seen in *Homarus americanus* (McLeese, 1973). In our experiments preferential turning to the intact side and circus movements (Table II) were only seen when laterally ablated lobsters also had the propodus and dactylus of their walking legs coated with glue. Such coated and ablated lobsters also showed greater search path errancy compared to lobsters with only lateral flagellum ablations (Table II). These results demonstrate that leg receptor input does play a secondary role in spatial orientation in a chemical stimulus field; this role becomes apparent when antennular chemoreception is disrupted. However, in otherwise intact lobsters, leg chemoreceptor input was not essential for efficient orientation.

The possibility of chemically stimulated rheotaxis can not be overlooked. Under this hypothesis, a lobster is stimulated by the biologically significant odor of food to search with mechanoreceptors in the ever-present flow gradients. In general, the chemical senses are closely allied with mechanoreceptors, both morphologically and functionally.

In animals such as lobsters, which regularly lose appendages to predators, in social interactions, or through molting disturbance, overlap and redundancy of sensory input must be very important. Our results indicate that when the main chemosensory input to spatial orientation is lost, other inputs can take over, at least partially. This behavioral recovery of function was not complete in the short duration of these experiments (days), but may well improve over time (weeks) as suggested by studies on hermit crabs (Hazlett, 1971) and lobsters (Atema *et al.*, 1981).

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# SOMATOTOPY IN THE REPRESENTATION OF THE PECTORAL FIN AND FREE FIN RAYS IN THE SPINAL CORD OF THE SEA ROBIN, *PRIONOTUS CAROLINUS*

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## Abstract

Sea robins possess modified pectoral fin rays which are chemosensory although lacking taste buds or olfactory receptors. These fin rays are innervated only by spinal nerves which terminate in accessory spinal lobes, enlargements of the dorsal horn in the rostral spinal cord.

Horseradish peroxidase was used as a neuronal tracer to determine the representation of each fin ray nerve in the spinal cord. The nerve innervating each fin ray terminates in a single accessory lobe with the ventral fin ray terminating in the caudal accessory lobe and the dorsal fin ray in the rostral major accessory lobe. The pectoral fin itself is represented in a minor spinal enlargement lying rostral of the major accessory lobes.

## Introduction

Sea robins (*Prionotus*) and the related European gurnards (*Triglida*) possess modified pectoral fins which are capable of detecting low levels of certain chemicals despite the absence of taste buds or other specialized chemosensory end organs (Whitear, 1971). In these genera, the three most ventral fin rays are free from the rest of the pectoral fin (Fig. 1). The free fin rays are moved independently of the fin and are used actively to explore the substrate (Morrill, 1895; personal observation). The fish will respond positively when the free fin rays contact food or food extracts (Bardach and Case, 1965).

Despite the fact that the free fin rays are used to locate food, the fin rays possess no taste buds (or olfactory receptors), and are innervated only by spinal nerves (Morrill, 1895). Whitear (1971) confirms that no specialized chemosensory end organs occur on the fin rays although numerous isolated chemosensory cells lie in the skin. The fin ray nerves emerge from the fused dorsal root ganglion of the second and third spinal roots (Herrick, 1907; also see Fig. 2). The nerves innervating the pectoral fin proper also emerge from this same ganglionic mass.

At the level of entrance of these nerve roots into the spinal cord, three major paired accessory spinal lobes occur on the dorsal aspect of the spinal cord. Herrick (1907) describes these accessory lobes as enlargements of the dorsal horn of the spinal cord. In addition, smaller swellings occur farther rostral in the spinal cord. Because the numbering system used by Herrick does not correspond to the patterns of lobulation observed in the live specimens (to which Herrick did not have access),

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Abbreviations: HRP, horseradish perioxidase; HY, Hanker-Yates peroxidase method; TMB, tetramethyl benzidine.

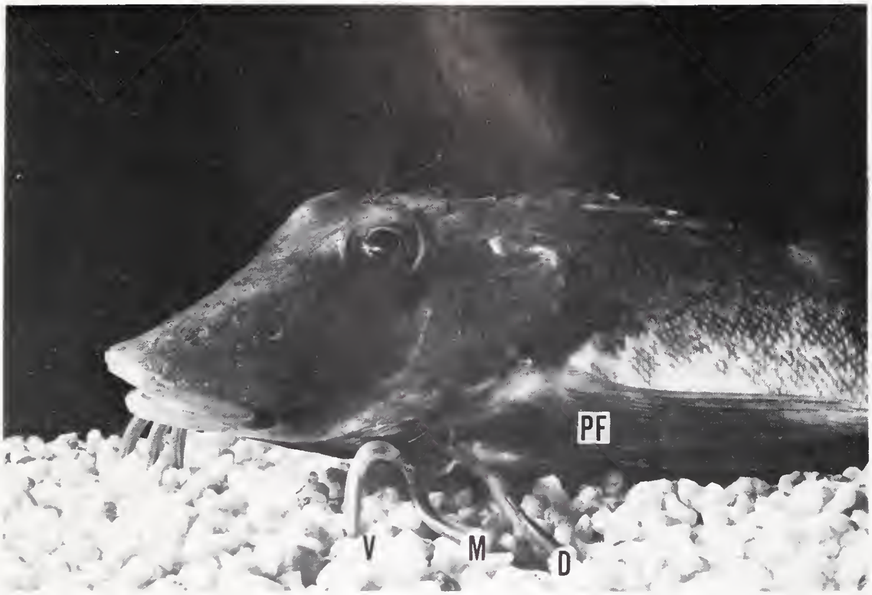

FIGURE 1. Photograph of a sea robin showing the free fin rays and pectoral fin. D, dorsal fin ray; M, middle fin ray; PF, pectoral fin; V, ventral fin ray. Approximately ⅔ life size.

the lobes have been renumbered in this work, according to the scheme indicated in Figure 2. The major accessory lobes are numbered 1–3, with number 1 being the most caudal. Accessory lobe 4 is a much smaller swelling located immediately rostral to the major accessory lobes. For reasons given elsewhere (T. Finger, in preparation) and below, the other swellings on the surface of the rostral spinal cord should not be considered homologous to the accessory lobes 1–4 described above and are not simple elaborations of the dorsal horn of the spinal cord.

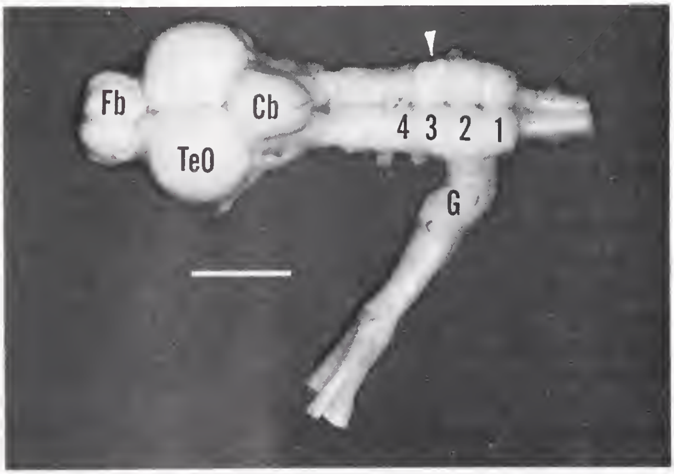

FIGURE 2. Photograph of the brain and spinal ganglion of a sea robin. White arrowhead indicates the sulcus of the accessory lobe wherein a blood vessel lies. 1, 2, 3, 4 indicate the accessory lobes of the spinal cord. Cb, cerebellum; Fb, forebrain; G, dorsal root ganglion for the fin ray nerves; TeO, optic tectum. Bar scale equals 5 mm.

The pathways and nuclei of the central nervous system involved in the spinal chemical sense have not been studied with modern anatomical techniques. This first in a series of papers on the common chemical sense in sea robins utilizes neuroanatomical tracing techniques to determine the representation of the pectoral fin and free fin rays in the rostral spinal cord. The pectoral fin nerves project to the minor accessory lobe (number 4) while the free fin rays project to the major accessory lobes (numbers 1–3).

## Materials and Methods

Live sea robins (*Prionotus carolinus*) were obtained through the collection service at the Marine Biological Laboratory, Woods Hole, MA. The animals were fed pieces of squid and maintained in holding tanks supplied by running water.

Operations were carried out on fish which were anaesthetized with tricaine methane-sulfonate (MS-222). Initially, the animals were placed in seawater containing a 1:10,000 dilution of the drug. When opercular movements were barely perceptible or had ceased, the fish was transferred to an operation chamber which held the animal semirigidly by means of modelling clay blocks. The fish was covered with wet gauze and a recirculating pump supplied water to a tube inserted in the animal's mouth. The water in the operating chamber contained anaesthesia at a dilution of 1:20,000–1:40,000 depending on the anticipated duration of the procedure; higher concentrations were necessary for the longer operations. Following the surgery, the fish was placed in its home tank and revived by placing its mouth over the inlet tube for incoming seawater.

Horseradish peroxidase (HRP, Sigma Type VI) was used as an anterograde or transganglionic tracer. A 30–50% solution of HRP was prepared in a 1% solution of $\alpha$-lysophosphatydal choline (lysolecithin). The tracer was applied in one of two fashions. For ganglionic injections, HRP paste was applied to the end of a size 00 insect pin which was then inserted repeatedly into the ganglionic mass (Finger, 1976). For applications to a peripheral nerve, the nerve was exposed and isolated on gelfoam. A small piece of gelfoam soaked in HRP was then placed on the cut end of the nerve. Fine forceps and an insect pin were used to divide the nerve into numerous fascicles which then were threaded through the HRP-gelfoam. In some cases, flattened sheets of styrofoam (from a hot-cup) were sandwiched around the nerve-gelfoam and the entire assembly glued (cyano-acrylate glue; histo-acryl or Crazy Glue®) back into place beneath the skin. The overlying skin was then sutured and glued to form a watertight covering.

A total of 12 fish was used in this study, but because some fish had two nerves labeled, one on each side, these animals represent 18 experiments. In four cases, the dorsal roots were labeled by intraganglionic injections of HRP. These cases provided the clearest labeling of the primary afferent terminals. The remaining 14 experiments entailed applications of HRP to the peripheral nerve as follows: ventral fin ray, three cases; middle fin ray, three cases; dorsal fin ray, four cases; superior pectoral fin nerve, two cases; inferior pectoral fin nerve, two cases.

Following survival times of 1–2 days for ganglionic injections (four cases) and 4–10 days for peripheral nerve injections (eight animals, 14 nerves), the fish were reanaesthetized and perfused through the conus arteriosus with 20 ml of saline followed by 50–100 ml of 4% glutaraldehyde in phosphate buffer (0.1 $M$, pH 7.2). The brain, rostral spinal cord, and, in some cases, spinal ganglia were removed from the animal, encased in gelatin (Finger, 1976) and fixed an additional 3–6 hours. The gelatin blocks were washed in phosphate buffer and stored overnight

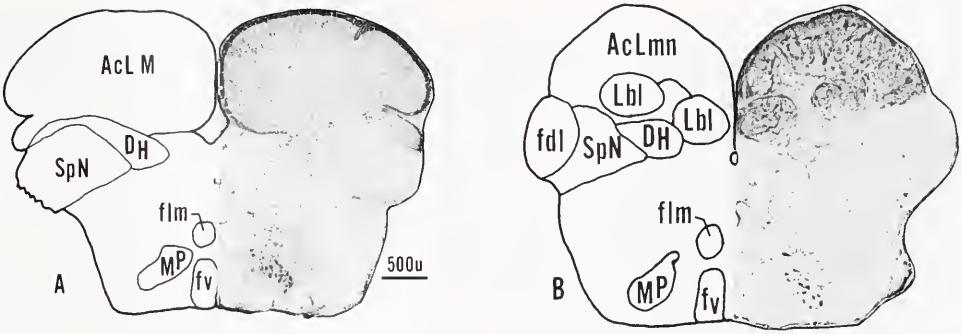

FIGURE 3. Photomicrographs and chartings of transverse sections through the accessory lobes. (A) Major accessory lobe. Note the prominent outer parvocellular layer surrounding the lobe. (B) Minor accessory lobe. The position of two lobules is indicated. AcLM, major accessory lobe; AcLmn, minor accesory lobe; DH, dorsal horn; Fdl, dorsolateral fasciculus; Flm, medial longitudinal fasciculus; fV, ventral fasciculus; Lbl, lobule; MP, segmental spinal motor neuron pool; SpN, spinal nerve root.

in buffer containing 10–20% sucrose. The tissue was sectioned at 35 μm in either the horizontal or transverse planes, on a freezing, sliding microtome. The sections were reacted for the presence of peroxidase by means of a modified Hanker-Yates (HY) method (Bell *et al.*, 1981) or by the tetramethylbenzidine (TMB) method of Mesulam (1978). In many cases alternate sections were reacted using the two different methods. The reaction product from the TMB method was visualized more easily, by darkfield microscopy, and the TMB reaction was slightly more sensitive. However, the HY method produced a less granular reaction product which better revealed fine structural details of the labeled fibers and cells.

## RESULTS

*Pattern of peripheral innervation.* The three free fin rays are each innervated by a unique branch arising from the fused ganglion of the second and third spinal roots (Morrill, 1895; Herrick, 1907). The fin ray nerves form separate fascicles within 1 cm of the ganglion, somewhat dorsal to the pectoral fin proper.

Two other major nerves leave this same ganglionic mass to innervate the pectoral fin proper. A large nerve turns rostrally from the ganglion and enters the pectoral fin from its superior, or anterodorsal, aspect. This nerve is termed the superior pectoral fin nerve. The smaller nerve innervating the pectoral fin, the inferior pectoral fin nerve, travels with the fin ray nerves caudal to the pectoral fin but turns rostrally to innervate the inferior face of the pectoral fin. The fin ray nerves continue ventrally to reach the free fin rays. Immediately before entering the fin ray, a given fin ray nerve splits into two branches, one branch innervating the surface rostral to the cartilagenous core of the fin ray, and one branch innervating caudal to the cartilagenous core. No attempts were made in the present study to trace separate connections of the anterior and posterior branches of each fin ray nerve.

*Structure of the accessory lobes.* The major spinal accessory lobes (numbers 1–3) each are divided in half along their rostrocaudal axis by an indentation along the dorsal surface. This superficial groove often embraces a blood vessel. No similar division of the minor accessory lobe (lobe 4) occurs although blood vessels do run across the surface of this structure as well.

All of the accessory lobes contain an outer layer (approx. 40 μm thick) of small neurons and an inner zone of neurons mixed with neurophil (Fig. 3A). Golgi prep-

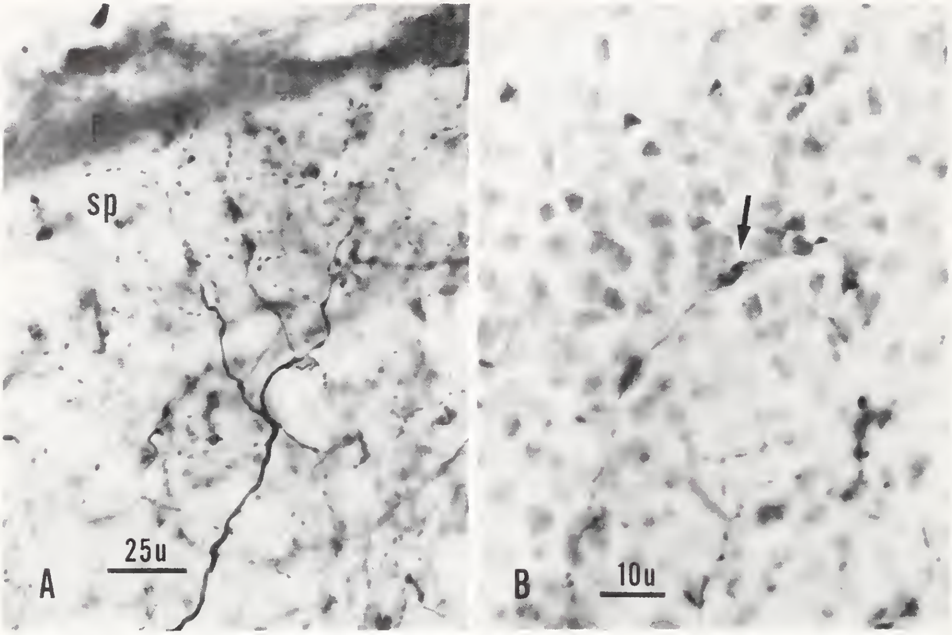

FIGURE 4. Photomicrographs of terminals and preterminal arborizations in the spinal accessory lobes following intraganglionic injection of HRP. Hanker-Yates reaction. (A) Numerous branches and *en passant* swellings occur in the subparvocellular layer (sp). p, parvocellular layer. (B) An apparent terminal among the small neurons of the parvocellular layer.

arations reveal that most dendrites of neurons in the accessory lobes are oriented radially. The detailed structure of the lobes will be discussed in a later paper (T. Finger, in preparation). The minor accessory lobe (lobe 4) comprises a number of lobules, each of which is surrounded by a parvocellular layer which extends inward from the surface of the lobe (Fig. 3B).

*Primary afferent fibers.* The morphology of the primary afferent fibers is revealed clearly by ganglionic injections of peroxidase. The nerve roots enter the spinal cord from its lateral aspect; in the case of lobe 4, the root has a slight rostral inclination as it penetrates the cord. A few root fibers terminate in the dorsal horn beneath the accessory lobes. The vast majority of the primary afferent fibers enter the accessory lobes from below and turn radially outward to terminate throughout the substance of the lobe. A given primary afferent fiber may branch repeatedly in its course outward through the lobe. Numerous terminal swellings occur throughout the lobe, however a heavier band of terminal arborization appears in the outer 50 $\mu$m of the neuropil of the lobe, *i.e.* immediately subjacent to the superficial parvocellular layer (Fig. 4A). A few terminal swellings are scattered amongst the somata in the parvocellular layer (Fig. 4B), but the bulk of the terminals and *en passant* swellings occur in the subjacent neuropil. This pattern of termination occurs in all the accessory lobes, minor as well as major.

*Somatotopic organization.* The central area of termination of each fin or fin ray nerve was determined by relying on transganglionic transport of HRP. This relatively fine, light labeling was revealed best by the TMB technique and darkfield microscopy (Fig. 5A) although the reaction product was clearly visible following reaction with the modified HY substrate.

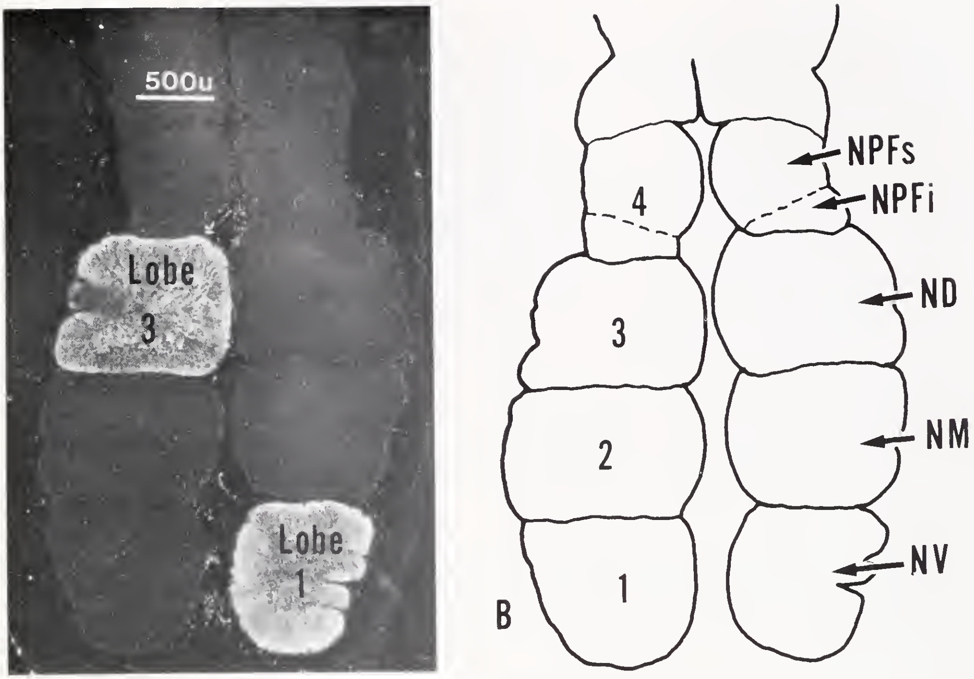

FIGURE 5. Horizontal section through the rostral spinal cord of a sea robin, anterior upward. (A) Darkfield photomicrograph from a case in which the dorsal fin ray nerve had been injected on the left side and the ventral fin ray nerve had been injected on the right side. Transganglionic transport of the peroxidase tracer shows in the photograph as a bright area (lobe 3 on the left and lobe 1 on the right). In these cases, no label appears in lobe 2 or the minor accessory lobe, above lobe 3. (B) Semischematic drawing of Fig. 5A showing the somatotopic representation of the various nerves in the rostral spinal cord. 1–4, accessory lobes; ND, dorsal fin ray nerve; NM, middle fin ray nerve; NPFi, inferior pectoral fin nerve; NPFs, superior pectoral fin nerve; NV, ventral fin ray nerve. Same scale as Fig. 5A.

Application of HRP to the nerve innervating the ventral fin ray resulted in labeling of terminals in lobe 1, the middle fin ray in lobe 2, and the dorsal fin ray in lobe 3 (Fig. 5). There was virtually no overlap between nerves in their projection onto the accessory lobes. The pectoral fin nerves terminate in the minor accessory lobe (lobe 4). The terminals from the inferior nerve occupy the caudolateral one-quarter of the lobe with the superior nerve terminals filling the remaining three-quarters of the structure (Fig. 5B). In summary, the central representation of the fin rays and pectoral fin is somatotopically organized. The ventral fin ray, farthest from the fin, is represented most posteriorly and the fin itself most anteriorly.

No primary afferent fibers ascend in the cord to reach the level of the caudal medulla. Therefore there does not appear to be a system in this species homologous to the dorsal columns found in amniote vertebrates.

## DISCUSSION

Sea robins use their free fin rays to explore their surroundings. That the fin rays are chemoreceptive has been demonstrated both by behavioral and electrophysiological means (Scarrer *et al.*, 1947; Bardach and Case, 1965). Since the fin rays lack taste buds and are innervated only by spinal nerves, this chemosensitivity has been attributed to the common chemical sense (Parker, 1922). Therefore at least some, if not most, of the fibers in the fin ray nerves mediate the common chemical sensibility. Compared to other spinal nerves, the pectoral fin and fin ray nerves are unique in terminating in the accessory lobes. Accordingly, the accessory lobes probably are involved in processing input from the common chemical sense.

The pectoral fin and fin rays are represented in a somatotopic order in the spinal cord. Somatotopy in a chemosensory system is not unique to this modality; a gustatory somatotopy has been described for catfish at the level of both the primary (Finger, 1976) and secondary (Finger, 1978) sensory nuclei.

One surprising result in this study is the order of the somatotopic map in the spinal cord. The ventral fin ray, which is also the most anterior part of the fin, is represented in the most posterior accessory lobe. The pectoral fin, which lies posterior (and dorsal) to the fin rays is represented farther anteriorly in the cord. If the pattern of innervation reflects the dermatome of origin in the embryo, then this implies that the anteroventral part of the fin, including the fin rays, arises posterior to the rest of the fin. If so, this further implies that the fin has rotated during embryogenesis such that the posterior edge of the fin in the embryo moves ventrally and rostrally during development so as to lie anterior and ventral to the rest of the fin in the adult.

The description of the accessory lobes given in this study is not identical to the descriptions offered by Morrill (1895), Ussow (cited in Morrill, 1895), or Herrick (1907). These authors describe six accessory lobes; the present report describes four. This difference is attributable to the more detailed study of intrinsic morphology and connections given in the present report. Four morphologically similar accessory lobes are described herein and they correspond to the accessory lobes 2–6 of Herrick (1907) which are identical to the five caudal lobes (unnamed) illustrated by Morrill (1895). Both of these authors divide accessory lobe 2 of this study into two parts on the basis of the sulcus in which the lobar blood vessel lies (see above). However, upon careful examination (see Fig. 2), all the major accessory lobes are marked by a similar sulcus. Since the portions of each lobe rostral and caudal to this sulcus are indistinguishable both in terms of morphology and connections, there is no apparent reason to separate these two halves of the same structure. In addition to the doubling of this central major accessory lobe (lobe 2), both Herrick and Morrill describe another accessory lobe lying rostral to the minor accessory lobe (lobe 4 of this study). This more rostral structure receives input predominantly from descending primary afferent fibers of the trigeminal nerve (T. Finger, unpub. obs.). As such, this structure is similar to the medial funicular nucleus described by Herrick (1906) and Finger (1976). Furthermore the morphology of this medial funicular nucleus in sea robins (Herrick's accessory lobe 1) is quite different from that of the accessory lobes proper. The medial funicular nucleus lacks the external cell layer which is characteristic of the accessory lobes. Accordingly, the present study does not include the medial funicular nucleus among the spinal accessory lobes.

The minor accessory lobe (lobe 4) receives input from the nerves innervating the pectoral fin which itself is supported by numerous fin rays. One possible explanation for the lobules in the minor accessory lobe is that each fin ray of the pectoral fin is represented in a single lobule. This conjecture needs to be tested by either finer anatomical or electrophysiological experiments. Furthermore, since the morphology of the minor accessory lobe is similar to the major lobes, the pectoral fin itself may be capable of chemoreception albeit with less sensitivity or discriminability than the free fin rays. Whitear (1971) reports that isolated chemosensory cells are scattered throughout the epidermis of many, if not all, teleosts. Accordingly, the fin ray chemosense may represent a specialization of a spinal chemosense present in many vertebrates (Parker, 1922).

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# MORPHOLOGICAL AND BEHAVIORAL IDENTIFICATION OF THE SENSORY STRUCTURES MEDIATING PHEROMONE RECEPTION IN THE BLUE CRAB, *CALLINECTES SAPIDUS*

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## ABSTRACT

Scanning electron microscopy was used to survey the aesthetasc tuft on the outer flagellum of the antennule (1st antenna) in order to identify sensilla potentially involved in pheromone detection by the male blue crab. These studies showed that the tuft of each antennule is divided into a mesial and lateral half by a region of cuticle from which no sensilla arise. Two setal types were revealed: the aesthetascs and previously undescribed sensilla which originate exclusively on the mesial side of the tuft and project to the lateral half between the rows of aesthetascs. Experiments were performed in which the mesial half, lateral half, or entire aesthetasc tuft was bilaterally ablated from the antennules of test males. As revealed by behavioral tests, pheromone responses in "mesial half" and "lateral half" ablation groups were reduced 22% and 21%, respectively, relative to control ($P > 0.10$); whereas a highly significant ($P < 0.005$) response decrement (80% relative to control) occurred in the "entire tuft" ablated group. The data suggest that pheromone reception in the male blue crab is effected via the aesthetascs. The relationship of these findings to those for other decapod crustaceans is discussed.

## INTRODUCTION

Previous work demonstrated the presence of a pheromone in the urine of pubertal females of *C. sapidus* which triggers courtship behavior in males (Gleeson, 1980). It was further shown that detection of this pheromone occurs via chemoreceptors located on the outer flagellum of the antennules (first antennae) as indicated by the lack of courtship responses for males in which the outer flagella were ablated.

In the present study the outer flagellum was examined using scanning electron microscopy (SEM) to identify structures potentially involved in pheromone reception. This effort focused on the aesthetasc tuft region since these sensilla are presumed to be chemosensory ("olfactory receptors") in decapods (Ache, in press). Based on the morphological information, various lesions were made in the tuft and any decrement in pheromone response was assessed behaviorally.

## MATERIALS AND METHODS

### *Morphology*

Antennules were treated in Karnovsky's fixative for two to two and a half hours, rinsed in 0.1 *M* sodium cacodylate buffer, and dehydrated through a graded ethanol

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series. The samples were then transferred to acetone, subjected to critical point drying, and, after gold coating, examined with a scanning electron microscope.

To evaluate the permeability of structures on the outer flagellum, the crystal violet technique of Slifer (1960) was utilized. Antennules were fixed in a 10% solution of formalin for 24 hours, then rinsed in water and exposed to a 0.5% solution of crystal violet for periods varying from five seconds to 10 minutes. After two rinses in distilled water the specimens were dried, cleared in xylene, and mounted for inspection under the light microscope.

### *Ablation experiments*

All studies were performed during the summer months using the facilities at the University of Maryland's Marine Products Laboratory in Crisfield, Maryland. Animals were obtained locally from commercial sources, held in tanks (1.2 × 2.4 × 0.3 m) with a flow-through water system, and sustained on a diet of fish.

A test-tank (1.0 × 1.0 × 0.2 m) in which the water depth was maintained at 10 cm via a standpipe drain was used for all behavioral testing. Water filtered to 10 $\mu$m was introduced to one corner of the tank at a rate of approximately five liters per minute. As a source of pheromone, three to six pubertal females (those within six days of undergoing their maturity molt) were retained in an acrylic cylinder (15 cm height by 30 cm diameter) which was positioned close to the inflow corner of the test-tank.

In each trial six male crabs were introduced to the test-tank immediately following placement of the females within the cylinder. The activity of the males was observed over a five minute period after which the position of the inflow delivery tube was switched to overflow the water in the cylinder. The actions of the males were then noted over a second five minute observation period and courtship responses recorded as defined previously (Gleeson, 1980). The criteria used to identify courtship behavior were:

1) A courtship display—chelae extended in the lateral position, swimming appendages (fifth pereopods) rotated anterodorsally and waved from side to side above the carapace, and walking legs (second to fourth pereopods) extended such that the body is elevated to a near maximum height above the substrate; or

2) An approach towards another test-male with chelae extended in the lateral position, followed by an attempt to cradle-carry the approached individual.

All males were pre-tested in the apparatus, and only those exhibiting courtship responses were used for ablation treatments. These treatments involved bilateral antennule operations performed under a dissecting microscope. The crabs were restrained and each antennule held in position by a clamp mounted on a micromanipulator which allowed positioning the antennule such that the aesthetasc tuft was accessible for ablation. Four treatment categories were examined using randomly selected males:

1) *Ablation of the entire aesthetasc tuft.* Water was blotted from the tuft and the hairs manipulated from their normally recumbent position to allow cutting the entire tuft with micro-dissecting scissors.
2) *Ablation of the mesial portion of the tuft.* Again, this involved blotting the tuft and manipulating the hairs from their recumbent position. Fine-tipped forceps were used to pluck all of the hairs from the mesial half of the tuft, leaving the aesthetascs of the lateral half intact.

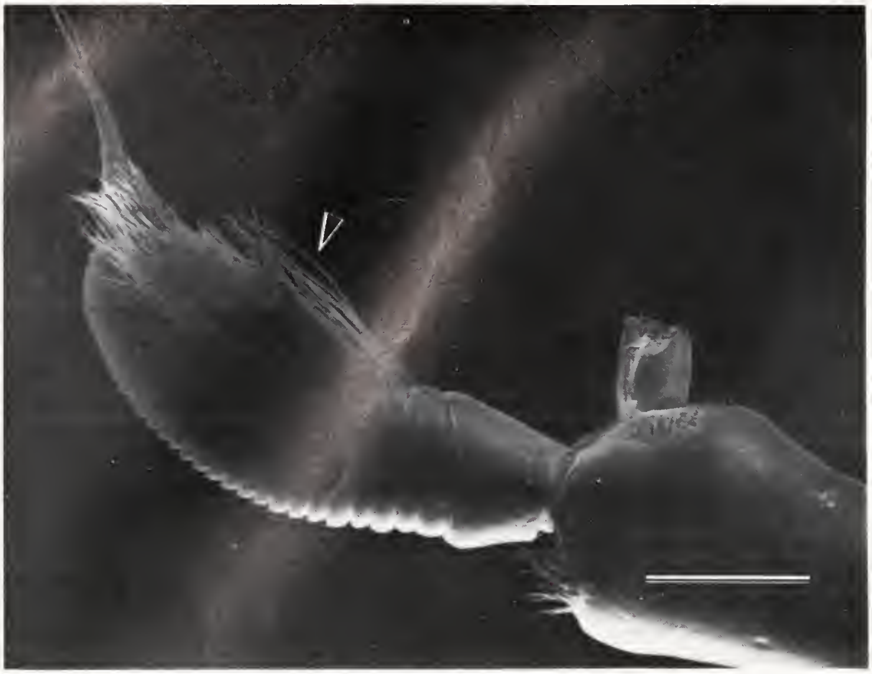

FIGURE 1. Lateral view of antennule tip showing aesthetasc tuft (arrowhead) on outer flagellum. Inner flagellum was removed. Scale bar = 700 $\mu$m.

3) *Ablation of the lateral portion of the tuft.* The procedure was as in (2) except that the lateral half of the tuft was removed and the mesial portion left intact.
4) *Sham control.* This process was as for all of the above treatments, but with no cutting or plucking of hairs within the tuft.

Between 24 to 48 hours after ablation treatments the males' pheromone responses were tested. In order to reduce the incidence of false negatives (*e.g.*, lack of response due to the stimulus failing to reach receptor sites), each male was examined in up to three trials. Two untreated control males were simultaneously tested with treated animals in every trial, and any trial in which none of the six males responded was voided.

At the conclusion of the behavioral tests, the antennules of the treated males were removed and prepared for SEM inspection.

## RESULTS

### *Morphology*

The outer flagellum of the antennule (Fig. 1) is approximately 2 mm in length and characterized by a series of over 30 segments which give it flexibility. A prominent feature of this flagellum is the tuft of approximately 650–700 aesthetasc hairs which arise from grooves distally situated on the ventral surface of most flagellar segments (Fig. 1 and 2A). This tuft is divided into mesial and lateral halves by a central region of cuticle from which no aesthetascs arise (Fig. 3A). The aesthetasc setae are from 700–1000 $\mu$m in length and approximately 10–12 $\mu$m in diameter. Three to five distinct bulges (Fig. 3B) are characteristic of the proximal region of

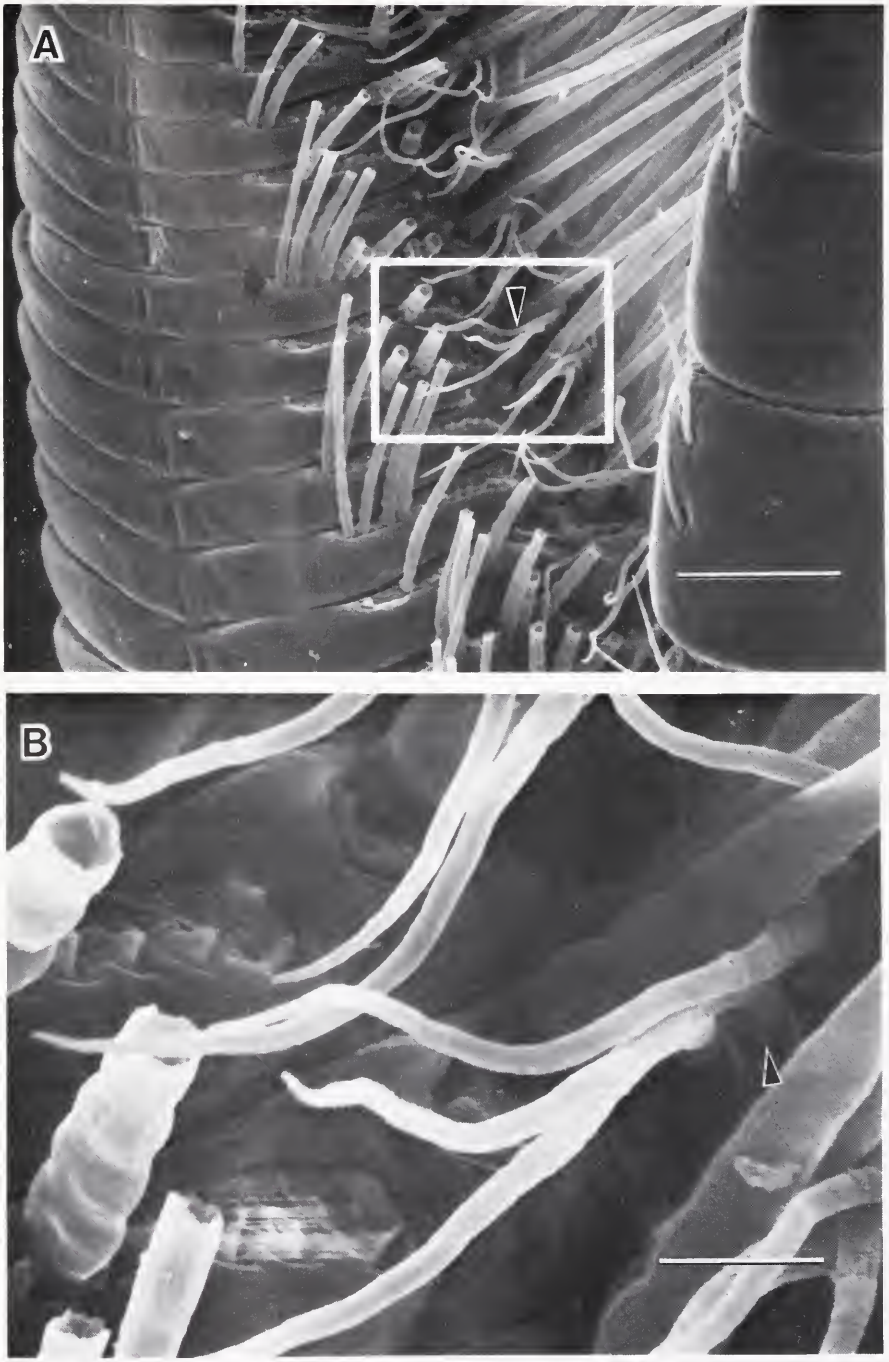

FIGURE 2. (A) Ventro-lateral view of tuft region. Lateral half of tuft was removed allowing visualization of the asymmetric sensilla (arrowhead). Aesthetascs (broken) in groove on the distal border of a flagellar segment are indicated by the arrow. Scale bar = 135 $\mu$m. (B) 5× magnification of the enclosed region in (A). Arrowhead indicates socket location of an asymmetric sensillum on mesial side of tuft. Scale bar = 27 $\mu$m.

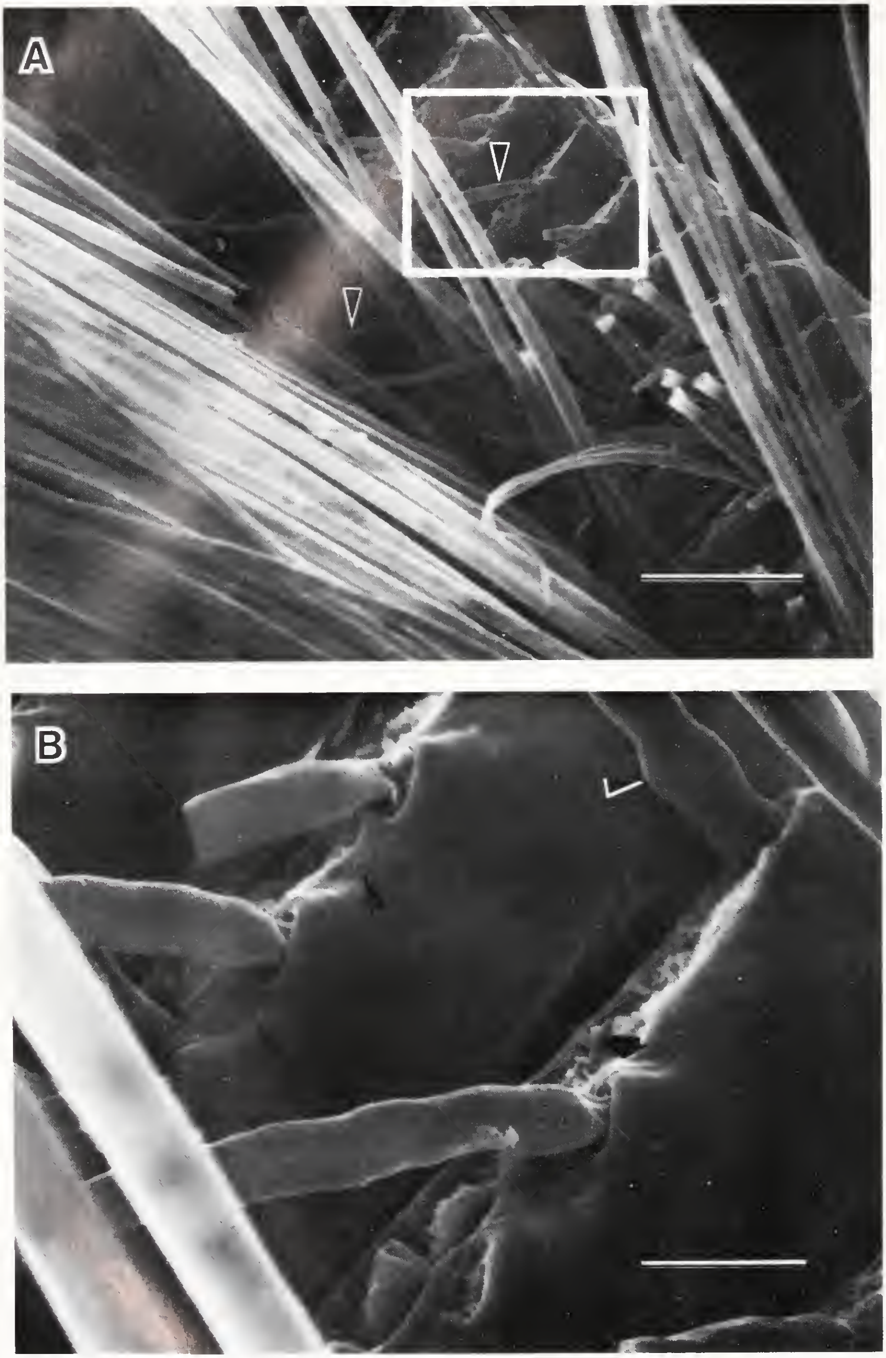

FIGURE 3. (A) Ventral view of tuft in which a portion of the mesial half was removed. Note central region of cuticle lacking sensilla (arrows). Asymmetric sensilla indicated by arrowheads. Scale bar = 100 $\mu$m. (B) 5× magnification of the enclosed region in (A). Arrow indicates socket of asymmetric sensillum. Black arrowheads show pore structures in cuticle. Annular bulge in basal region of aesthetasc indicated by black on white arrowhead. Scale bar = 20 $\mu$m.

each aesthetasc, and these give way to periodic annulations (about 30 μm apart) for the remainder of the hair shaft until the seta tapers to a tip, approximately 2 μm in diameter, which lacks a terminal pore.

Confined to the mesial half of the tuft, and proximal to the aesthetasc row of each segment, are groups of sensory hairs (0–4 per flagellar segment) with an external morphology unlike that of the aesthetascs. These setae, herein referred to as asymmetric hairs because of their exclusively mesial origin, arise from sockets which project from the cuticle at an angle such that the hairs extend across the tuft from the mesial to the lateral side (Fig. 2A, B and 3A, B). The asymmetric setae range in length from 170 to 220 μm, with diameters between 6 and 8 μm at the base, tapering gradually to a 1 μm tip with no terminal pore. Numbers of these hairs range from 46 to 70 per flagellum.

The only other surface features in the tuft region are small pores (0.3–0.6 μm in diameter) which are distributed along the distal portion of each flagellar segment just proximal to the groove from which the aesthetascs arise (Fig. 3B). Accurate counts of these structures are lacking, but the numbers range on the order of 20 to 60 per segment. Observations using light microscopy revealed that the pores are openings of canals extending 3–4 μm through the cuticle from spherical chambers (approximately 3 μm in diameter) which are situated on the inner surface of the cuticle. The aesthetasc tuft is the only region of the antennule in which these pore structures are found.

Permeability studies using crystal violet showed that both the aesthetascs and asymmetric setae were penetrated within five seconds. The asymmetric hairs were stained along their entire length, whereas a differential penetration occurred in the aesthetascs. The basal region of each aesthetasc (that section in which the annular bulges are located) was less darkly stained than the remaining portion of the hair, even after 10 minutes of exposure.

Concurrent SEM studies using antennules from females revealed no obvious sexual dimorphism in numbers or morphological types of sensory structures on the outer flagellum.

### *Ablation experiments*

As revealed by SEM, more than 90% of the setae in the targeted regions of the aesthetasc tuft were removed or otherwise lesioned in nearly all experimental animals (Fig. 4 and 5). The results of these studies are graphically depicted in Figure 6. Removal of the mesial and lateral portions of the aesthetasc tuft produced nearly equal reductions in the response levels of males. These reductions are not statistically significant, however, when compared to the control group using a Chi-square evaluation ($P > 0.10$). In contrast, for males in which the entire tuft was ablated, the response decrement is highly significant ($P < 0.005$) when compared to any of the other treatment categories. These latter data are further supported by an additional group of seven males in which the aesthetasc tufts were similarly ablated; all were unresponsive to pheromone stimulation when examined in two trials each.

## DISCUSSION

This study provides evidence which, in conjunction with previous morphological, behavioral, and physiological work, corroborates the postulated chemosensory function of the aesthetasc setae (see for example Laverack, 1964; Ghiradella *et al.*, 1968; Hazlett, 1971). Specifically, the data establish that these setae are of critical importance to the male *C. sapidus* in detecting the pheromone of the pubertal

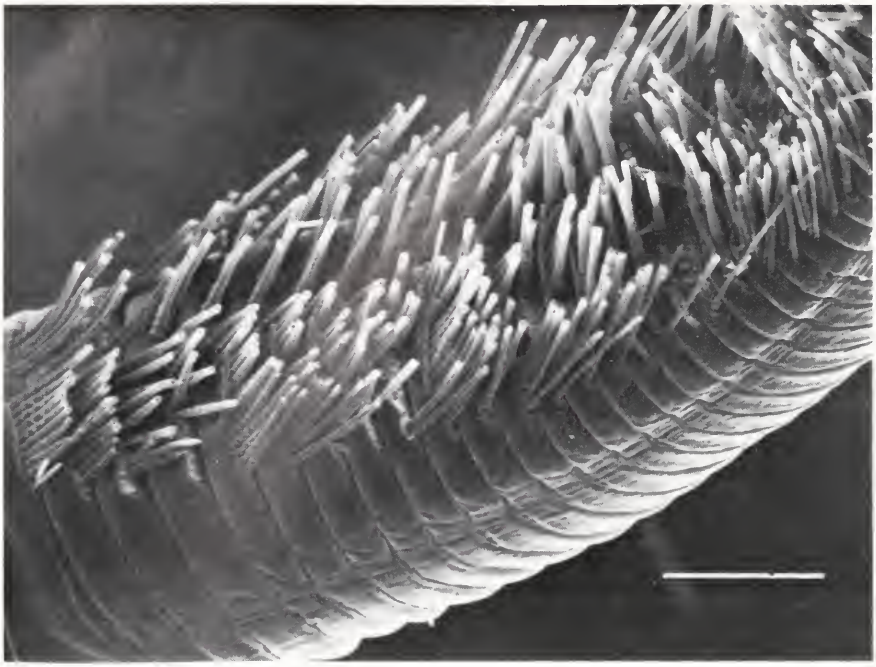

FIGURE 4. Ventro-mesial view of tuft region in which setae were cut with micro-dissecting scissors. Scale bar = 200 $\mu$m.

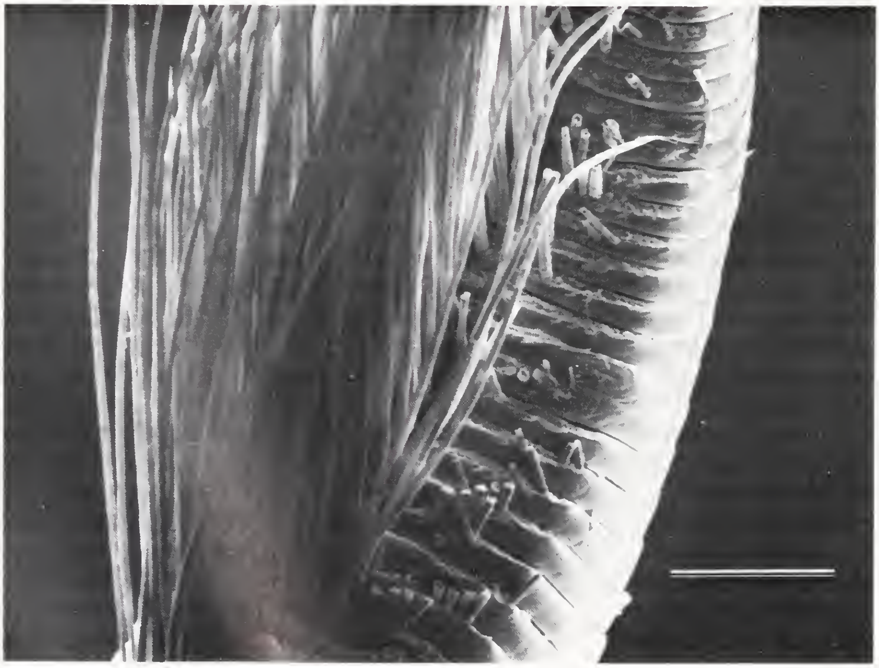

FIGURE. 5. Ventro-mesial view of tuft region in which mesial half was removed using forceps. Scale bar = 200 $\mu$m.

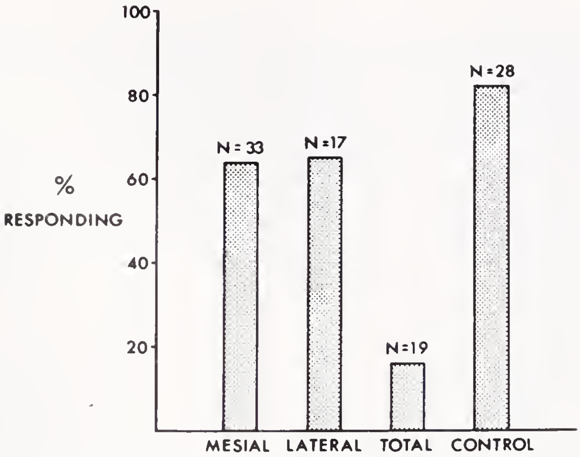

FIGURE. 6. Courtship responses of males in which the mesial half (mesial), lateral half (lateral), or entire (total) aesthetasc tuft was ablated from both antennules. Sham control (control) procedure was as for other treatment groups, but with no removal of setae. N = number of animals tested.

female, thus extending the findings of earlier experiments which localized this detection to the outer flagellum of the antennules (Gleeson, 1980).

Cutting the tuft (entire tuft ablation treatment) reduced the length of the aesthetascs such that less than one third remained intact, and this procedure decidedly blocked pheromone detection in males so treated. On the other hand, despite lesions to half the tuft, the animals in the mesial and lateral ablation groups retained their ability to detect the pheromone and also appeared equally competent in this detection capability, albeit at a reduced level relative to control. This implies that the receptors required for pheromone recognition are common to both halves of the tuft. If convergence of primary sensory neurons onto second order olfactory cells is involved in amplifying a pheromone signal (van Drongelen *et al.*, 1978), the reduced response level in these treatment groups might therefore reflect an increase in detection threshold resulting from loss of half the peripheral input to second order cells.

SEM inspection of the aesthetasc tuft region revealed relatively few sensory structures as compared, for example, to *Pagurus* (Snow, 1974) and *Panulirus* (Laverack, 1964). Other than the aesthetascs the only setae in this zone are the asymmetric hairs which are confined to the mesial half of the tuft. Their removal clearly did not alter pheromone detection in test males as indicated by the equally responsive mesial and lateral ablation groups. They are quite permeable to crystal violet, however, suggesting a possible chemoreceptive role, but this remains to be determined physiologically. Extracellular recordings from axons of cells innervating the asymmetric hairs revealed that these structures are at least mechanosensory. Deflection of the hair using a fine glass probe elicits phasic bursts of action potentials (Gleeson, unpublished data). The orientation of the asymmetric setae (*i.e.*, projection across the tuft between the rows of aesthetascs) in conjunction with this preliminary physiological data suggests that these structures may serve to monitor water flow through the tuft, such as would occur during flicking of the antennule (Schmitt and Ache, 1979).

The significance of the pores located exclusively in the tuft region is an intriguing unknown. Similar structures have been found associated with the aesthetascs of

*Homarus americanus* (Atema, 1977; Derby, 1982) and with certain setal types on the antennae of the sergestid shrimp, *Acetes sibogae australis* (Ball and Cowan, 1977). However, since data on the underlying structure of these pores is incomplete, speculation as to their function must await further study.

The question of pheromone receptor location has been addressed at various levels in other crustaceans. For *Palaemon paucidens*, Kamiguchi (1972) reported that the inner branch of the bifurcated outer flagellum of the antennule is relatively longer in males than in females and has a greater number of sensory (presumably aesthetasc) hairs. Although no experiments were conducted to test the hypothesis, it is postulated that this dimorphism is related to sex pheromone detection on the part of the male as has been found to be the case in many insect species (Schneider, 1964). A similar sexual dimorphism in the quantitative distribution of aesthetascs has been reported for several other crustacean groups as well (Barber, 1961).

Christofferson (1970) noted that removal of the aesthetasc-bearing outer flagellum [erroneously labeled the inner flagellum in that study and uncorrected in Dunham's (1978) review] from the antennules of the male *Portunus sanguinolentus* blocked the behavioral response to the female's sex pheromone. This response was not affected in control animals in which the inner flagellum (mislabeled the outer flagellum) was removed.

Based on ablation and electroantennulogram studies, Ameyaw-Akumfi and Hazlett (1975) and Ameyaw-Akumfi (1976) concluded that the inner (non-aesthetasc bearing) flagellum of the antennule in the male crayfish, *Procambarus clarkii*, contains chemoreceptors mediating sex recognition. The evidence on which this conclusion is based, however, is not entirely convincing. Although it is stated that test animals were unresponsive following removal of the inner flagellum, no data are presented for evaluation. Furthermore an important control condition is lacking: namely, an examination of test animals following ablation of the outer flagellum. Since the physiological data do not contribute to a resolution of this issue, the potential role of the aesthetascs in sex recognition by *P. clarkii* remains uncertain. Indeed, the situation is further confounded by the experiments of Itagaki and Thorp (1981) who used a flow-through design to examine chemical communication in *P. clarkii* and found no evidence for chemically mediated sex recognition in this species.

Dahl *et al.*, (1970a, b) present evidence suggesting pheromone reception in *Gammarus duebeni* occurs via calceoli which are male-specific sensory structures located on the second antennae. Their hypothesis is based on the apparent binding of a female-specific natural product to the calceoli, as demonstrated in males exposed to water in which radiolabeled females were retained. Recent work by Lyes (1979) has supported this hypothesis: "masking" or ablating the second antennae of the male *G. duebeni* forestalls pairing with females. However, Hartnoll and Smith (1980) found it necessary to remove both the first and second antennae to significantly block pairing; indicating that recognition of premolt females can be mediated via sensory structures other than the calceoli.

In summary, the information to date identifying structures mediating pheromone reception in decapod crustaceans is fairly limited and in some cases requires further experimentation. The present study implicates the aesthetascs as important receptors for sex pheromone detection in *C. sapidus*, but whether these structures prove to generally function in this capacity for decapods must await future comparative work specifically addressing the role of these setae in sex recognition.

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# NEW VICTORELLIDS (BRYOZOA, CTENOSTOMATA) FROM NORTH AMERICA: THE USE OF PARALLEL CULTURES IN BRYOZOAN TAXONOMY

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## ABSTRACT

Three species of the Victorellidae (Bryozoa, Ctenostomata) were found in North America and were cultivated in Braunschweig (W. Germany). Two are new species, *Victorella pseudoarachnidia* sp. nov. and *Tanganella appendiculata* sp. nov. *Bulbella abscondita* Braem is reported for the first time from Massachusetts (U. S. A.). Identification of victorellids requires the examination of living animals. The species considered here were identified by observation of live material collected in North America and Germany and of living individuals cultured from that material.

## INTRODUCTION

Kent (1870) described a species of the bryozoa (class Gymnolaemata, order Ctenostomata) as *Victorella pavida*, based on material collected in brackish waters in England. Kraepelin (1887) concluded that some specimens interpreted primarily as immature *V. pavida* (from the Ryck River, Germany) represented a new species and named it *Paludicella mülleri*. Braem's (1911) first opinion was that *P. mülleri* was only a developmental stage of *V. pavida.*

After many years of observation of living victorellids from northern Germany, Braem (1951) split the literature species *Victorella pavida* into three species. After a comparison with preserved *V. pavida* from England, Braem designated certain specimens to be *V. pavida*, characterized by the seasonal production of an intertentacular tube, the absence of brooded embryos, and the location of the cardiac sphincter far above the central stomach. Victorellids which produced no intertentacular tube, brooded embryos internally, and possessed a cardiac sphincter near the central stomach were placed into a new genus as *Tanganella mülleri.* Specimens characterized by shorter peristomial tubes, a small intertentacular tube, external brooding of embryos, and the cardiac sphincter moderately above the central stomach were assigned to a new genus and new species, *Bulbella abscondita.*

Although these distinguishing characteristics were adequately described by Braem (1951), Brattström (1954) expressed some doubt about Braem's splitting of *V. pavida.* Brattström apparently overlooked the important fact that, unlike Braem, most workers described new species from preserved specimens. Preserved ctenostomes, however, do not usually exhibit all features necessary for identification. The characteristics described by Braem (1951) have been confirmed on living specimens from other localities (Jebram, 1969, 1976). These studies on living victorellids, then, suggest that many specimens identified from preserved material may have been other species.

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Another facet of the taxonomic problem in the Victorellidae is evident in several North American reports. Osburn (1944) studied *Victorella pavida* from the Chesapeake Bay (Atlantic Coast, U. S. A.) and reported that the colonies collected from the upper (less saline) end of the bay tended to be less branched. However, his photograph (1944, Pl. V) of *V. pavida* shows brooded embryos and therefore suggests, according to Braem's (1951) work, that at least a part of Osburn's material was not *Victorella.*

Rogick (1949) described *Nolella blakei* from Woods Hole, Massachusetts (Atlantic Coast, U. S. A.) and compared it to other ctenostomes. Soule (1957) identified specimens from the Salton Sea (California, U. S. A.) as *N. blakei* and *V. pavida.* He incorrectly synonymized *Tanganella mülleri* with *V. pavida*, but did not discuss the major distinguishing characteristics used by Braem (1951). Rogick (Soule, 1957) confirmed Soule's identification of the preserved material. Examination of Soule's Salton Sea specimens revealed victorellids but no *Nolella* (Jebram, personal observations, 1977).

Hyman (1959) was one of the few zoologists who acknowledged Braem's (1951) results, but she misinterpreted his comments on a crucial point. She (1959, p. 333) wrote that "brooding arrangements vary greatly, even within the same genus . . .", and she misquoted Braem in her statement that in *V. pavida* "each egg as it emerges from the supraneural pore is caught in a depression of the adjacent dorsal body wall, and eventually passes into the coelom of the dorsal side of the vestibule where three or four developing embryos may be found." These comments and her figures 125C and 125D apply not to *V. pavida* but to *T. mülleri. Victorella pavida* produces an intertentacular tube (not a simple pore) and does not brood embryos (Braem, 1951; Jebram, 1969, 1976).

Subsequent investigators essentially ignored Braem's (1951) findings and continued to use predominantly external characteristics for the identification of the victorellids (Prenant and Bobin, 1956; Sacchi and Carrada, 1962; Carrada and Sacchi, 1964; Everitt, 1975; Poirrier and Mulino, 1977). Osburn (1944) and Soule (1957) synonymized various forms with *V. pavida.* Osburn (1944) and Everitt (1975) independently suggested that branching, the formation of adventitious zooids (another external characteristic), correlates positively with salinity. Poirrier and Mulino (1977) confirmed this correlation in 49 of 52 samples but suggested that "other factors . . . may also influence branching."

The confusion evident in taxonomic and ecological papers on *Victorella pavida*, the relatively poor condition of *V. pavida* specimens in the British Museum (Jebram, Everitt, personal observations, 1980), and the apparent lack of any specimens of *Nolella blakei*, including Rogick's material from Woods Hole (Everitt), led to the current investigation. During the fourth conference of the International Bryozoological Association in Woods Hole, Massachusetts (U. S. A.) in September of 1977, we discussed some problems in victorellid taxonomy. The purposes of this investigation were to collect *N. blakei* from the type locality, to collect victorellids from the Salton Sea (California, U. S. A.), to culture any victorellids collected, and to revise the taxonomy of these North American victorellids on the basis of observations of living animals.

## MATERIALS AND METHODS

In September of 1977 and in August of 1978, victorellids were collected from the bridge pilings at the outlet of Lagoon Pond on Martha's Vineyard Island (Mass., U. S. A.), the type locality for *Nolella blakei.* Living colonies were observed and were either preserved in 10% formalin or cultured in the laboratory (Braunschweig, W. Germany). In October of 1977, victorellids were collected in the Salton Sea (California, U. S. A.) and were treated likewise.

The methods for culturing bryozoans were described previously (Jebram, 1977a, 1977b, 1979). The methods for culturing victorellids were developed anew according to earlier results with other bryozoan species (Jebram, 1980b). For comparative morphology the victorellids were maintained at 21–23°C and 14–16‰ salinity and were fed the food mixture J5b, the composition of which was determined experimentally. The descriptions of the species provided here are based on specimens cultivated under these conditions (Jebram, 1980b). Details on morphology and techniques are given under "Experimental Biology".

After the animals attained sexual maturity, they were compared with living victorellids collected in Germany and with earlier descriptions based on living specimens. Cultures of *Victorella pavida*, *Tanganella mülleri*, and *Bulbella abscondita* have been maintained in the laboratory of the senior author (*T. mülleri* since 1968). Colonies subcultured from the holotype specimens of the American victorellids discussed here are being maintained for further study; Louisiana (U. S. A.) specimens are also being cultured in Braunschweig.

Drawings of the species described here were prepared by Jebram from photographs of living animals. Paratype specimens are in the collections of the authors.

## SYSTEMATIC TREATMENT AND RESULTS

### Victorella pseudoarachnidia *sp. nov.*

Holotype material: Collection of D. Jebram, 1978-10-10-1.

Paratype material: U. S. National Museum of Natural History, Smithsonian Institution (Washington, D. C.), Cat. No. 36, USNM No. 292472; Bryozoan Collections of the Allen Hancock Foundation, Univ. of Southern California at Los Angeles, No. 185.1; British Museum (Natural History), London; personal collections of authors.

Name: The cystid appendages suggest superficial similarities to an arachnidioid form.

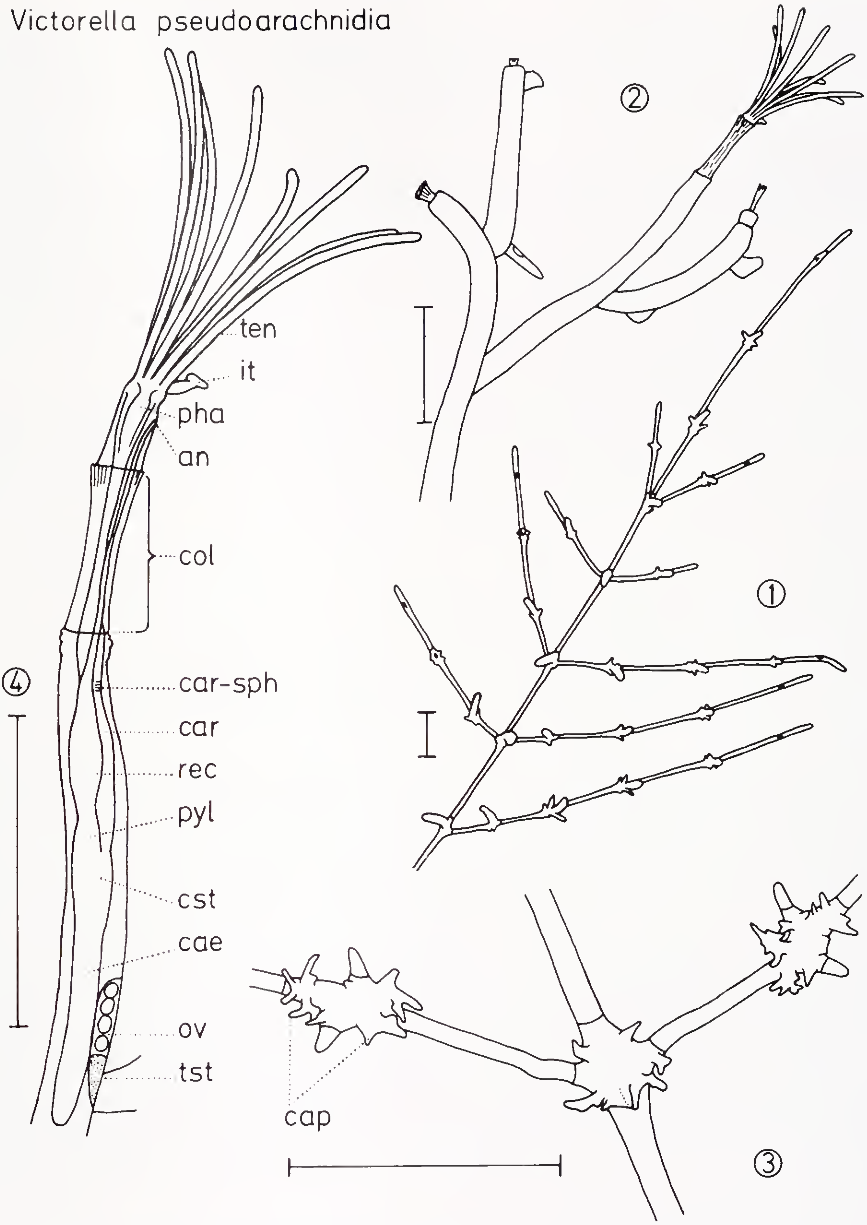
Synonyms: "*Victorella pavida* Kent" and "*Nolella blakei* Rogick" *sensu* Soule, 1957 (nec. *Victorella pavida* Kent, 1870, *sensu* Braem, 1951; nec *Nolella blakei* Rogick, 1949).

Type locality: Salton Sea, California, U. S. A.

Description: The colonies of *Victorella pseudoarachnidia* are composed of serially arranged zooids (Fig. 1). Each zooid usually produces one distal and two lateral daughter zooids at the sides of the basal part of the cystid. The branching pattern of these daughter zooids is regular and symmetrical. Older zooids can produce adventive zooids by forming "high buds" ("Hochknospen") on the anal or lateral sides of the peristomial tube (Fig. 2). In addition to the encrusting colony parts ("forma encrustans" *sensu* Braem, 1951) are often free, non-encrusting branches ("forma ascendens" *sensu* Braem, 1951) of zooid series formed at the borders of the substrate pieces or originating from the high buds. The colony in this species thus has a habit somewhat similar to that of most other victorellids.

Each zooid is composed externally of two main parts, a basal proximal part (usually encrusting the substrate) and an upright peristomial tube. The basal part is broad at its distal end and narrow proximally; the proximal narrowing usually is relatively abrupt.

Encrusting zooids of *V. pseudoarachnidia* have cystid appendages which usually originate on each side of the budding sites of the daughter zooids. The basic pattern



FIGURES 1–4: *Victorella pseudoarachnidia* sp. nov. (1) part of the holotype colony demonstrating the budding pattern in an encrusting colony; (2) "high buds" and adventive zooids in lateral view; (3) three zooids in basal view demonstrating the budding pattern and the cystid appendages; (4) sexually mature zooid in lateral view; an, anus; cae, caecum; cap, cystid appendages; car, cardia; car-sph, cardiac sphincter; col, collar; cst, central stomach; it, intertentacular tube; ov, ovary; pha, pharynx; pyl, pylorus; rec, rectum; ten, tentacles; tst, testis; scale bars represent 1 mm.

consists of 9–10 sites for the potential formation of cystid appendages (Figs. 3, 11). All cystid appendages may branch weakly into two to several tips. One or a few of the distinct appendages may be vestigial or absent. The branching of the appendages may occur under the central basal part of the cystid so that only the tips project visibly beyond the lateral margin of the cystids. The non-encrusting zooids form no (or vestigial) cystid appendages. The formation of cystid appendages is generally variable and somewhat modifiable but never occurs on the narrow, most proximal part of the basal cystid. The origin of all the tips of the cystid appendages from restricted and rather distinct sites of a zooid can often be detected only if the animals are growing on glass slides and are illuminated sufficiently. Otherwise the shape of the central basal cystid parts may superficially resemble an arachnidioid form. In the victorellids, the appendages of different zooids may touch each other but never really fuse histologically as they can in typical arachnidioid forms.

The peristomial tubes grow stepwise upward by each case of polypide replacement and may thus attain a considerable length (to about 1 cm with polypide retracted). The diameter of the peristomial tube is about 220 μm.

The autozooids of *Victorella pseudoarachnidia* always have 8 tentacles. Sexually mature zooids have a trumpet-shaped intertentacular tube with a widely flared opening, through which the ova are released (Fig. 4). This species does not brood embryos, either in the neck region or in the tentacle sheath. Freshly released ova are spindle-shaped and whitish and become globular after approximately fifteen minutes.

The gut anatomy of this species shares two essential attributes with that of *Victorella pavida*: location of the cardiac sphincter considerably above the central stomach (Fig. 4) and absence of a gizzard.

*Victorella pseudoarachnidia* produces lasting buds which may have irregularly shaped marginal appendages and which, in older stages, have a greyish or dark brown to almost black cuticle. The storage products in the ova and in the lasting buds are whitish.

The accompanying fauna in the type locality of *V. pseudoarachnidia* included the stolonate ctenostome *Bowerbankia cf. gracilis* Leidy and the kamptozoan (entoproct) *Barentsia benedeni* (Foettinger) (Jebram, field observations, 1977). Soule (1957) did not report these species from his Salton Sea samples.

Differentiating characteristics of similar forms: (1) In other species of *Victorella* described so far, there has been no mention of the formation of such types of branched cystid appendages at restricted sites of the zooids. (2) Species of *Tanganella* differ in the location of the cardiac sphincter near the central stomach, the formation of an intertentacular pore, the brooding of embryos, and the form and arrangement of the cystid appendages. (3) Almost all species of the superfamily Arachnidioidea have the potential to produce cystid appendages, but these appendages are usually narrow and rarely branched and may originate also from the narrow proximal part of the cystid. In the Arachnidioidea the appendages and branches may actually fuse histologically and may produce new zooids at the fusion sites (Fig. 11).

### Tanganella appendiculata *sp. nov.*

Holotype material: Collection of D. Jebram, 1978-10-10-2.

Paratype material: U. S. National Museum of Natural History, Smithsonian Institution (Washington, D. C.), Cat. No. 36, USNM No. 292473; Bryozoan Collection of the Allan Hancock Foundation, Univ. of Southern California at Los Angeles, No. 186.1; British Museum (Natural History), London; personal collections of authors.

Name: The cystids usually have appendages at restricted sites.

Synonyms: part of the material of *Victorella pavida sensu* Osburn (1944), according to his description (nec. *Victorella pavida* Kent, 1870, *sensu* Braem, 1951).

Type locality: Lagoon Pond, Martha's Vineyard Island, Massachusetts, U. S. A.

Description: The colony of *Tanganella appendiculata* is composed of serially arranged zooids (Fig. 5). Each zooid usually produces one distal and two lateral daughter zooids on the sides of the basal part of the cystid. Older zooids can form adventive zooids by producing "high buds" on the anal and lateral sides of the peristomial tube (Fig. 6). In addition to the encrusting colony parts (forma encrustans) free, nonencrusting branches of zooid series often may be formed at the borders of substrate pieces or may originate from "high buds" (forma ascendens). The colony form of this species thus resembles that of most other victorellids.

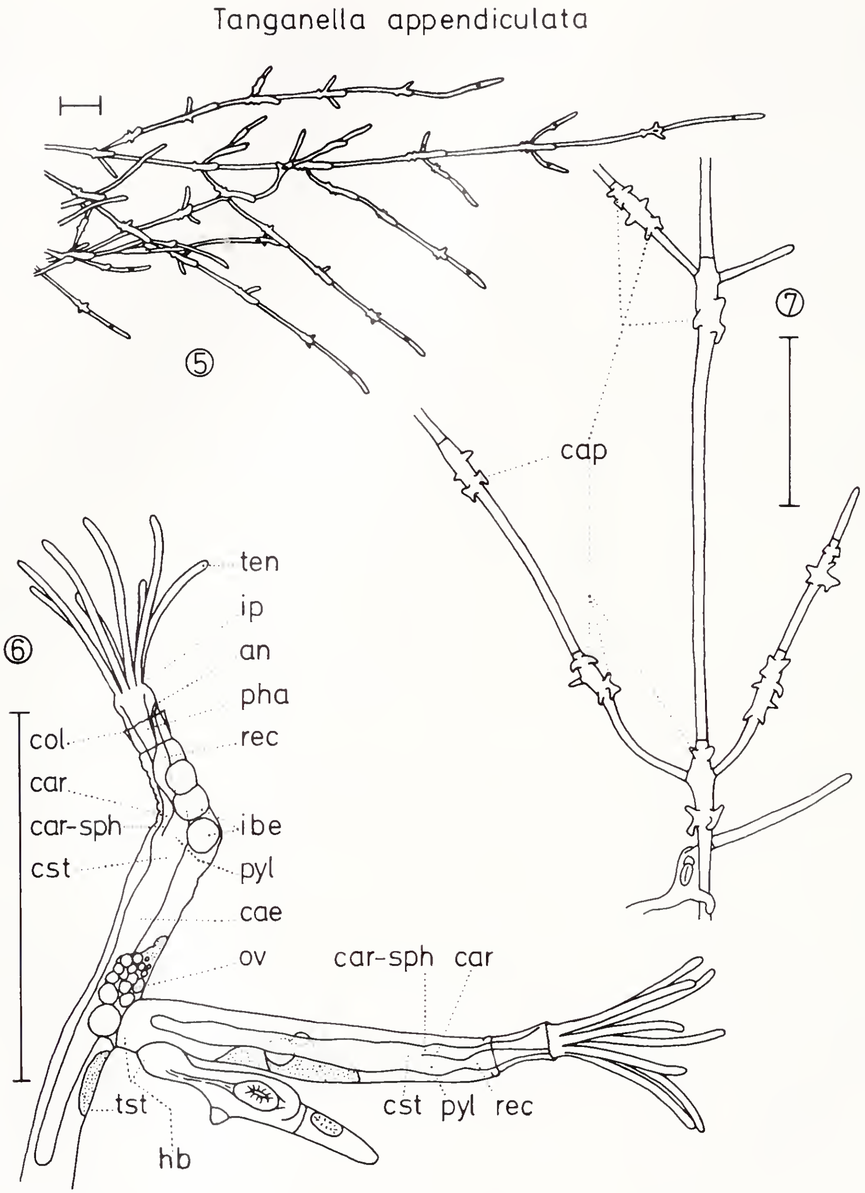
The zooids are composed externally of two main parts, a basal proximal part (usually encrusting the substrate) and an upright peristomial tube. The basal part is somewhat broadened at its distal part and gradually narrows proximally.

Encrusting zooids of *Tanganella appendiculata* usually have typical cystid appendages. One or two emerge, usually latero-proximally on each side, at the sites from which the lateral daughter zooids originate from the basal part of the cystid. One or two other pairs of cystid appendages may be formed anterior to the budding places of the side-branches (Fig. 7). However, the cystid appendages, especially the distal pair, are sometimes vestigial or absent. The appendages may be so minute that they are detectable only if the zooids are growing on glass slides and are adequately illuminated. These appendages, of course, can be overlooked easily in specimens from the natural habitat, especially if they are encrusting a rugged substrate. The cystid appendages sometimes branch into several tips and even into four separate appendages. In this species, the cystid appendages always originate from the sides of the distal, broader portion of the encrusting part of the cystid, never from the narrow, proximal portion. Non-encrusting zooids produce no cystid appendages (or only vestigial ones). The appendages from different zooids may touch each other but never fuse histologically.

The younger peristomial tubes are always considerably inclined distally; older ones may become raised almost perpendicular to the basal part of the cystid. The peristomial tubes grow upward in steps by each case of polypide replacement in the same cystid and may attain a length of approximately 9 mm (polypide retracted). The diameter of the peristomial tube averages 160 $\mu$m.

The autozooids of *Tanganella appendiculata* always have 8 tentacles. Sexually mature zooids have an intertentacular pore through which the ova are released. This very narrow pore is discernible in living animals only during the release of the egg through the pore.

The ova are apparently fertilized during their passage through the intertentacular pore. At that time they are dumbbell-shaped or irregularly shaped but not spindle-shaped. The released ova are pressed to the anal neck region, where they adhere to the body wall. Later they are invaginated into a pocket of the body wall; the embryos remain there until they develop into larvae (Fig. 6). Up to six embryos may be brooded in the median line of the anal neck region of one zooid. The polypide apparently remains active throughout the period of ova release (several days) but later may be resorbed. The larvae slip through the breaking body wall into the water and may swim for several hours or days (even more than 10 days!) until they find an acceptable place for settlement. The first polypide of the ancestrula of *Tanganella appendiculata* has 6 tentacles.


Tanganella appendiculata
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FIGURES 5–7: *Tanganella appendiculata* sp. nov. (5) part of the holotype colony demonstrating the budding pattern in an encrusting colony; (6) sexually mature zooid with adventive zooid and "high buds," hb, in lateral view; (7) some zooids in basal view demonstrating the budding pattern and the cystid appendages; ibe, internally brooded embryos; ip, site of the intertentacular porus; for other abbreviations see Figs. 1–4; scale bars represent 1 mm.

Lasting buds are usually formed in larger colonies. The cuticle of older lasting buds becomes light brown by thickening. The reserves of the lasting buds, the ova, and the embryos are white.

The anatomy of the gut of *Tanganella appendiculata* is similar to that of *T. mülleri*. The cardiac sphincter is very close to the central stomach, a characteristic given by Braem (1951) for the genus. No gizzard is formed. The caecum is considerably longer and more slender than in *Victorella* in proportion to the size of the polypide.

The accompanying fauna in the type locality included *Bowerbankia gracilis* Leidy, *Barentsia benedeni* (Foettinger), and *Bulbella abscondita* (see below).

Differentiating characteristics of similar forms: (1) *Tanganella mülleri* (Kraepelin) *sensu* Braem (1951) forms 7 tentacles in the first polypide generation of the ancestrula. Braem (1951), who made most of his observations of colonies growing on natural (non-glass) substrates, did not describe cystid appendages in *T. mülleri*. These structures have now been detected under culture conditions for both species. Under certain salinity ranges and dietary conditions, *T. mülleri* may form comparatively smaller cystid appendages at 5 sites of a cystid, 2 latero-proximally and 3 distally from the budding sites of the side branches (Fig. 11). The appendages, however, especially the distal ones, are often absent or vestigial. In the main branches, the encrusting basal cystid parts are at least 30% shorter in *T. mülleri* than in *T. appendiculata* (under the same growth conditions), but this length may vary greatly in both species with external conditions. As Braem (1951) explained, contrary to the assumptions of various other authors, "*Paludicella mülleri*" respectively *Victorella pavida* forma *mülleri* in the sense of Ulrich (1926) is not *Tanganella mülleri* but *Bulbella abscondita*. Nevertheless, Prenant and Bobin (1956) ignored Braem's (1951) correction and erroneously maintained the incorrect identification and synonymy of Ulrich (1926). (2) *Victorella pseudoarachnidia* differs from *Tanganella appendiculata* in the location of the cardiac sphincter farther above the central stomach, in the formation of an intertentacular tube, in not brooding embryos, and in the form and arrangement of cystid appendages. (3) The species of the superfamily Arachnidioidea show the same differences as with *Victorella pseudoarachnidia* (Victorelloidea).

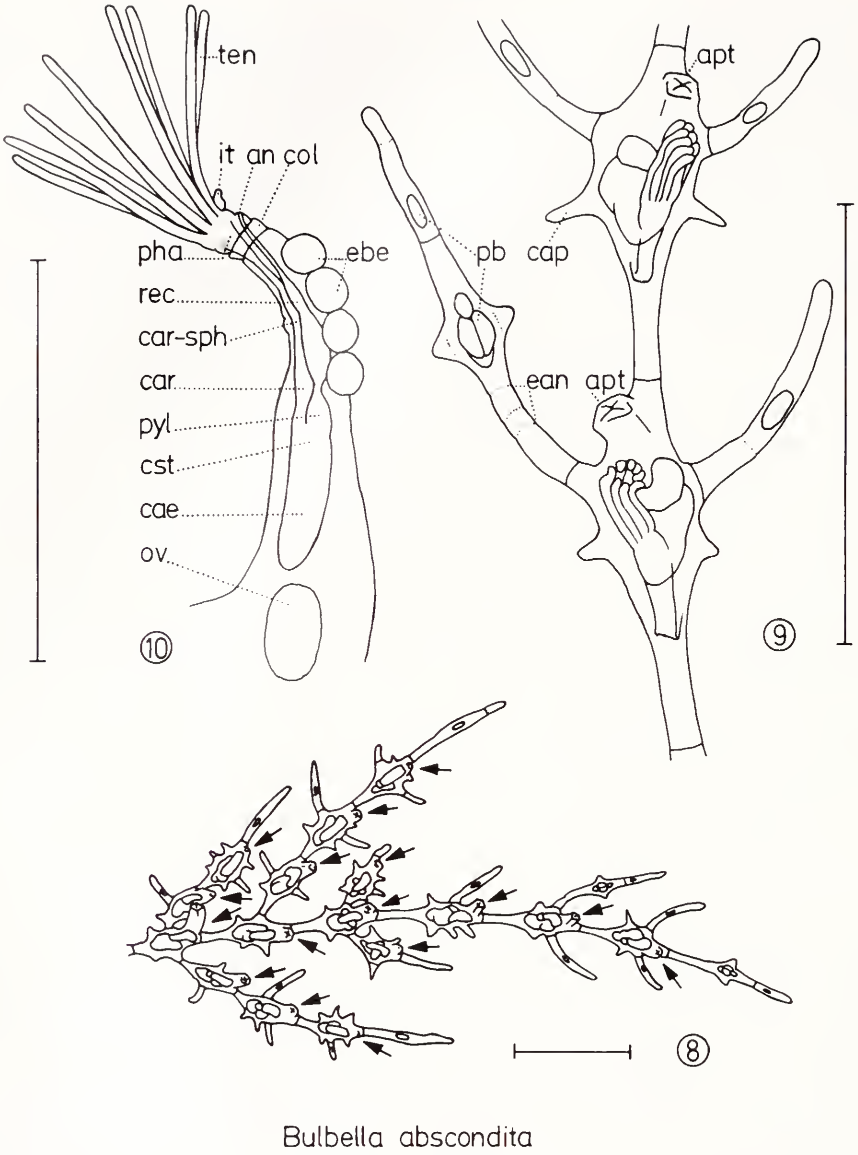
## Bulbella abscondita

Paratype material: U. S. National Museum of Natural History, Smithsonian Institution (Washington, D. C.), Cat. No. 36, USNM No. 292474; Bryozoan Collection of the Allan Hancock Foundation, Univ. of Southern California at Los Angeles, No. 187.1; British Museum (Natural History), London; personal collection of authors.

Locality: Lagoon Pond, Martha's Vineyard Island, Massachusetts, U. S. A.

Description: In most features the specimens found in this study resemble those described by Braem (1951). The colony is composed of serially arranged zooids (Fig. 8). Each zooid usually produces one distal and two lateral daughter zooids. Adventive zooids originating from high-buds occur rarely on older zooids. In old colonies, the zooids are crowded and grow irregularly. In addition to the encrusting zooids, the colony rarely may produce free, non-encrusting branches. The latter zooids occur mainly at the borders of the substrate but also may arise from the flat areas; their production is related partly to the diet.

The young zooids are comprised almost entirely of the basal cystid part, which is broader distally and slender proximally (Fig. 9). Although the polypide bud starts to develop as a median epidermal invagination (the usual process in ctenostomes), the aperture of the young encrusting zooids is always lateral. Within one



FIGURES 8–10: *Bulbella abscondita* (8) and (9) parts of the holotype colony demonstrating the budding pattern in an encrusting colony (arrows indicate the asymmetrical position of the apertural papillae); (10) sexually mature zooid in lateral view; apt, apertural papillae; ean, external annulations caused by too strong brushing; ebe, externally brooded embryos; pb, polypide buds; for other abbreviations see Figs. 1–4; scale bars represent 1 mm.

distally arranged zooid series, the right or left position of the initial apertures may vacillate irregularly, a kind of enantiomorphic effect. Only a small apertural papilla is formed in young zooids primarily with the first polypide generation. The replacement of the polypides in the same cystid causes a modest elongation of the peristomial tube, which becomes shifted to the median line in older, crowded zooids.

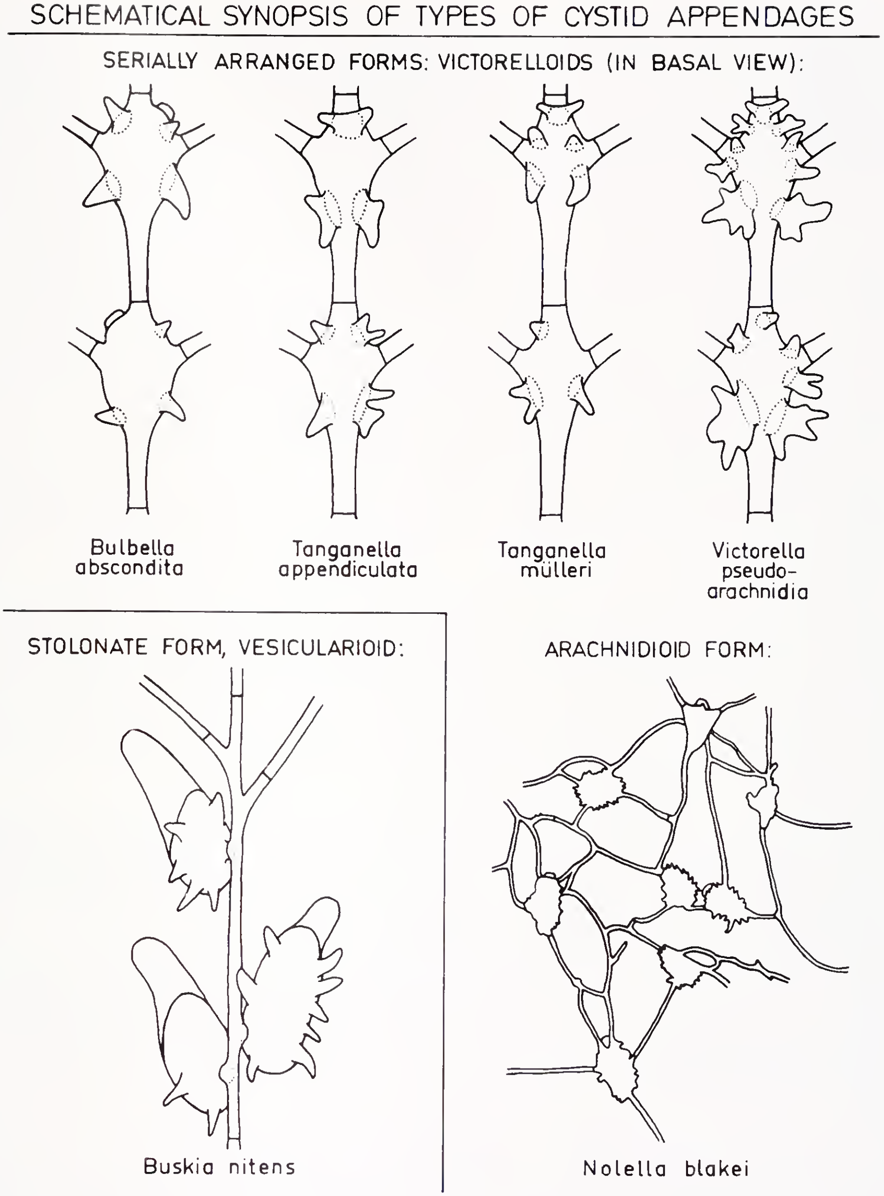

SCHEMATICAL SYNOPSIS OF TYPES OF CYSTID APPENDAGES
SERIALLY ARRANGED FORMS: VICTORELLOIDS (IN BASAL VIEW):
Bulbella abscondita
Tanganella appendiculata
Tanganella mülleri
Victorella pseudo-arachnidia
STOLONATE FORM, VESICULARIOID:
Buskia nitens
ARACHNIDIOID FORM:
Nolella blakei

FIGURE 11: Schematical synopsis of types of cystid appendages (in basal view). In the victorellid species, the upper zooid demonstrates the ground plan of the arrangement of the appendages, while the lower zooid shows an example of a more or less common arrangement. The sketch of *Buskia nitens* is an abstraction from various observations and published figures. The sketch of *Nolella blakei* is redrawn from Rogick (1949, fig. 5) but reversed in an assumed basal view. (All examples are drawn at different scales.)

The peristomial tubes may attain a length of approximately 3–4 mm and a diameter of approximately 260 μm.

Encrusting zooids of *Bulbella abscondita* often form cystid appendages on the sides of the broader, distal part of the basal region of the cystid but never on the narrower proximal part. Of four potential sites from which an appendage may emerge, 0–4 may actually produce one (Figs. 8, 9, 11); the number produced depends partly on diet conditions. These distinct sites for the cystid appendages are proximal and distal to the budding sites of the side-branches. The cystid appendages of different zooids may touch each other but do not fuse histologically.

The autozooids of *Bulbella abscondita* have 8 tentacles. Sexually mature zooids have an intertentacular tube with a narrow outlet through which the ova are released (Fig. 10). The ova are affixed to the ano-median line (sometimes to the ano-lateral side) on the polypide neck region. There the embryos (up to 6 per zooid) become larvae but are not invaginated into pockets of the body wall. The polypide of the mature zooid remains active during the ova-releasing period. The color of the reserves in the ova, the embryos, and the young larvae varies with diet and ranges from light yellow to almost white. The developed larvae are released and may swim for a few hours or days until they settle. The first polypide generation of the ancestrula has 7 tentacles (as in the typical form, Braem, 1951).

Some dormant buds, which were hidden under older zooids and were growing on glass slides, contained very light yellowish yolk and small polypide buds. The dormant buds found in this strain, however, have usually and essentially the shape of incompletely developed zooids but rarely of typical lasting buds found in other ctenostomes.

Braem (1951) stated that *Bulbella abscondita* usually has a gizzard with teeth but that the dentation may range from fully developed dentation to complete absence of teeth. In the North American specimens, a muscular proventriculus is present, but a true gizzard has not yet been observed. The cardiac sphincter is far above the central stomach and is more easily discernible in living animals during typical peristaltic movements of the gut. The caecum is comparatively short and stout. (For further details see Braem, 1951.)

Although there are some physiological differences between the North American strain and the Ryck (Germany) strain, the distinction is not yet sufficient to establish a separate species. We do not know whether the North American form can penetrate rotten wood as can the European form. The German form of *Bulbella* is being parallel-cultured in the laboratory at Braunschweig.

Differentiating characteristics of similar forms: (1) The species of the superfamily Arachnidioidea have the same differences as with *Victorella pseudoarachnidia*. (2) *Buskia nitens* has true stolons limited by septa. This species also prefers higher salinity (down to polyhaline) but never tolerates oligohaline conditions as does *Bulbella*.

## Experimental Biology

The discrimination of species in the Victorellidae requires living and sexually mature animals (Braem, 1951; Jebram, 1969, 1976). Two main factors controlling growth and attainment of sexual maturity in brackish-water bryozoans are nutrition and temperature (Jebram, 1973a, 1975). In North American victorellids discussed here, these factors, especially food, were investigated by various qualitative tests.

Based on prior studies of brackish-water bryozoans (Jebram, 1975, 1977b), diet composition for the new bryozoan strains was established by experience (Jebram, 1980b). The food mixture J5b (Jebram, 1980b) was prepared especially for the cultivation of *Bulbella abscondita* but is suitable also for other bryozoans. *Bulbella* becomes sexually mature with this diet. Under laboratory conditions, ova and embryos seemed to attain the typical light yellow color only by addition of those food species containing considerable amounts of carotenoids, *e.g.,* haptophyceans, chrysophyceans, bacillariophyceans. Some of the light yellow larvae produced with this diet metamorphosed successfully to ancestrulae and initiated the formation of a new colony generation. A diet mixture containing too much *Cryptomonas* species caused an earlier shifting of the apertural papilla from the cystid side toward the median line and an earlier and more pronounced elongation into a peristomial tube. The latter finding agrees with the observations made on *Bowerbankia* species and *Buskia nitens* (Jebram, 1973a, 1973b).

Although *Tanganella mülleri*, *T. appendiculata*, and *Victorella pseudoarachnidia* thrived and matured sexually with the food mix J5b, these species grew much better with a diet including *Oxyrrhis marina* (food mix J5h). This phagotrophic dinoflagellate is a very good food also for many other bryozoan species (Jebram, 1969, 1975, 1980a,b). Surprisingly, *Oxyrrhis*, presumably due to its taste, is very sparingly accepted by *Bulbella abscondita.* Therefore, *Oxyrrhis* should not be fed to *Bulbella* but may well be used for other victorellids. Diets containing *O. marina*, however, require a renewal at least each second day because the phagotrophic species soon alters drastically the composition of the diet preparation. Additionally, overaged cultures of *Oxyrrhis* may have toxic effects on the bryozoans (Jebram, 1975). If *Oxyrrhis* is used as a mono-food for a prolonged period, unusual growth forms may result. Further details concerning general problems of the nutrition of bryozoans have been discussed earlier (Jebram, 1977a,b, 1979, 1980a,b).

Even under the same external conditions (*e.g.,* food, salinity, temperature), all four victorellids cultivated in the Braunschweig laboratory (*Victorella pseudoarachnidia*, *Tanganella appendiculata*, *T. mülleri*, *Bulbella abscondita*) had different growth rates and formed different colony habits (qualitative observations). Although the different colony habits can be observed easily when the specimens are side by side, these differences can be described less easily. This difficulty was perhaps one of the reasons for the confusion in the taxonomy of this group of bryozoans in the past. *Victorella pseudoarachnidia* exhibited the fastest growth rate and formed larger bushes of the forma ascendens. The zoaria of *Tanganella appendiculata* colonized the substrate more quickly (by greater elogation of the narrow proximal cystid part) than those of *T. mülleri*, but the latter attained sexual maturity sooner. In *Victorella* and *Tanganella* the growth rate and the formation of adventive zooids by high buds were considerably greater, and sexual maturity occurred earlier with the food mixture J5h (with *O. marina*) than with J5b. *Bulbella abscondita* showed the slowest growth rate.

The formation of cystid appendages was apparently more or less influenced by unknown dietary components in all the victorellids cultivated. Additionally, in lower salinity ranges (5–8‰), *Tanganella mülleri* formed no (or only vestigial) appendages, but in *T. appendiculata* the appendages only became small (or were sometimes absent). With greater salinity (about 15‰) the formation of the appendages increased in both species of *Tanganella.* In *Victorella pseudoarachnidia*, however, the growth of cystid appendages seemed unaffected by variation of salinity within ecologically acceptable ranges.

*Tanganella mülleri*, *T. appendiculata*, and *Bulbella abscondita* inhabit areas in which the water temperature seldom reaches and rarely exceeds 20°C. Accordingly, these species attained sexual maturity in the laboratory at temperatures of 19°C or lower. On the other hand, the Salton Sea (California), from which *Victorella pseudoarachnidia* was collected, is in a warm semi-desert area (water temperature 26°C at 0900 on 9 Oct. 1977). Therefore this species must be adapted to higher temperature ranges. Accordingly, *V. pseudoarachnidia* seemed to require a temperature above 20°C for sexual maturation in the laboratory but grew well asexually at lower temperatures.

## Discussion

### *Cystid appendages in ctenostome taxonomy*

One reason for seeking ctenostomatous bryozoans in Lagoon Pond on Martha's Vineyard Island (Mass., U. S. A.) in September of 1977 was Rogick's (1949) report of *Nolella blakei*, which she thought lived in that pond. Although her specimens of *N. blakei* have not been found again, her description is undoubtedly that of an arachnidioid species. Instead of the expected species, we were surprised to find an obvious victorelloid, a species of *Tanganella*.

Rogick (1949) noted that she collected benthos from Lagoon Pond but that *Nolella blakei* was not seen initially in that material. She stored the material in watch glasses in large aquaria which were supplied with running sea water piped from the nearby bay. After nine days she discovered a ctenostome in those glasses and described *N. blakei*. It now appears that *N. blakei* does not inhabit the brackish Lagoon Pond but that it is a marine species (like most other species of *Nolella*) and that Rogick's colony originated from larvae in the seawater piped from the bay. Rogick did not mention any species of the Victorelloidea in her Lagoon Pond material.

The cystid appendages of *Tanganella appendiculata* were not observed in our first specimens from Lagoon Pond, in which they grew crowded with *Bowerbankia gracilis* on natural substrates. When the *Tanganella* material was cultured on glass slides in the laboratory at Braunschweig, the cystid appendages were detected. Another unexpected discovery was that the European *Tanganella mülleri* can also produce cystid appendages under certain conditions; Braem (1951) did not describe such appendages in *T. mülleri*.

These observations generated two basic questions. First, are there two separate species of serially arranged ctenostomes in the Salton Sea (California) as reported by Soule (1957)? The senior author examined Soule's specimens and, after additional studies on living animals, concluded that Soule's "*Nolella blakei*" is identical with his *Victorella* "*pavida*". Soule's specimens of "*Nolella*" were actually those zooids of *Victorella* which were growing on glass bottles. The cystid appendages on glass can be seen more easily than on other substrates and also can be removed more readily. Soule's *Victorella* "*pavida*" was mainly material from other types of substrate, and the cystid appendages were probably lost or damaged during removal of the zooids. One of Soule's drawings (1957, Fig. c) shows zooids of *V.* "*pavida*" with appendages, but the morphology of the appendages was not sufficiently analyzed. Such analysis requires proper procurement and preparation of specimens. The following features common to all known serially arranged ctenostomes from the Salton Sea indicate that they all belong to one species, a *Victorella*: constant number of tentacles (8); intertentacular tube; absence of brooding of embryos; anatomy of the gut; difference in arrangement and growth potential of cystid appendages as compared to arachnidioid species (see below).

The second basic question is whether the presence of cystid appendages in the true victorellids means that there are no principal differences between the Victorellidae and the Arachnidiidae. Such a separation has been doubted by some earlier authors. A close examination of anatomical details reveals the general differences between victorelloid appendages and arachnidioid appendages. In victorellids the appendages originate at distinct and more or less limited sites on the cystids, whether or not the potential appendages actually develop, and the appendages are never produced on the narrow and most proximal cystid part. In the Arachnidioidea, however, appendages may be formed irregularly at various sites of the cystid borders, including the narrow proximal cystid part. Appendages of different zooids may (but not necessarily) fuse histologically and often produce a new zooid at those points of fusion in arachnidioid species. This histological fusion does not occur in the victorelloid (and some stolonate) species (see Fig. 11).

In addition to observations of collected specimens, studies on living colonies of *Cryptoarachnidium argilla* have revealed the absolutely different growth potential of the arachnidioid cystid appendages. Banta (1967) originally described this species as *Victorella argilla*. Re-examination of paratype specimens showed that this species is undoubtedly not a victorelloid but an arachnidioid species, and the new genus *Cryptoarachnidium* was established (Jebram, 1973b). Specimens from Marina del Rey (California, U. S. A.) have been cultivated since October of 1977 in Braunschweig.

Cystid appendages apparently have developed independently in various phylogenetic lines in the Ctenostomata. Appendages are typical for most species of the Arachnidioidea but are formed also in several species of the Walkerioidea, *e.g.*, *Aeverrillia setigera* (Hincks), and the Vesicularioidea, *e.g.*, species of *Bowerbankia, Buskia*, and *Cryptopolyzoon*. This study has revealed that cystid appendages are common also in the Victorelloidea. In the latter superfamily, cystid appendages simply have been overlooked in some of the species in the past. The important characteristic for the placement of a species in a ctenostome superfamily, then, is not the presence or absence of cystid appendages but the details of their arrangement and growth potential.

### *Cultivation experiments as a taxonomic technique*

Rogick agreed with Soule (1957) that some of the Salton Sea specimens were *Nolella blakei*. The confusion of those workers resulted mainly from the fact that the available animals were already preserved and were sexually immature. Voluminous ecological surveys and monographs (*e.g.*, Schütz, 1963; Carrada and Sacchi, 1964) are less valuable if they are based partially or mainly on incorrect identification of some of the predominant primary consumers (bryozoans) in brackish habitats.

In microbiology and botany, experimental work as an aid for identification of species has a long tradition. In zoology, however, cultivation techniques for taxonomic purposes have been used for relatively few taxa, *e.g.*, protozoans, some polychaetes and platyhelminths, certain parasitic or pathogenic species, and, of course, for various genetics studies. Most taxonomists and most workers in faunistics and synecology traditionally study preserved specimens. Although Braem (1951) demonstrated the usefulness of living animals for some more delicate taxonomic problems, this approach has been virtually neglected in other earlier bryozoan studies. The results described above reveal that the taxonomy in the Victorellidae, including reports from Asia and Africa, can be sufficiently determined only on the basis of living animals and with parallel-culture methods under defined conditions. There should be no doubt that taxonomy in various groups of the Bryozoa (and other taxa) requires experimental work and detailed studies on living animals. In some cases, future taxonomic investigation must include not only morphological features already present but also the growth *potential* of the zooids.

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# THE RELEASE OF THE PEDAL DISK IN AN UNDESCRIBED SPECIES OF *TEALIA* (ANTHOZOA: ACTINIARIA)

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## ABSTRACT

Specimens presumed to belong to an undescribed species of *Tealia* were collected subtidally in the northeast Pacific. In contact with the asteroids *Dermasterias imbricata* and *Patiria miniata*, these animals expanded their oral disks, constricted their columns, and detached their pedal disks. Other asteroids had no such effect. Of five other species of *Tealia*, only *T. piscivora* showed similar behavior and only to *D. imbricata*. Electrophysiological records showed: 1) that *D. imbricata* evokes pulses in a slow conduction system (McFarlane's SS1); and 2) that a train of electrical stimuli also causes SS1 pulses and brings about the release. It is concluded that SS1 pulses trigger the releasing behavior of *Tealia* sp. as they do the release and swimming behavior of *Stomphia* spp. A review of pedal disk release in the actinians shows that it occurs only in certain genera and species in several families not closely related. Although the circumstances and functions of the release where known are not the same in different species, the neurophysiological mechanisms employed are strikingly similar. Also discussed are the active role of the pedal disk in special behavior patterns and a possible function of the release in the escape of *Tealia* sp. from a predator.

## INTRODUCTION

Sea anemones with adhesive pedal disks generally remain firmly fixed to the substratum. If they change positions at all they do so by extremely slow sliding steps across the surface. However, a few anemones detach their pedal disks rapidly in response to specific stimuli. *Calliactis parasitica* (Couch) does so in its symbiotic interactions with hermit crabs (Ross, 1967, 1974), and *Stomphia coccinea* (Müller) and *S. didemon* Siebert (Siebert, 1973) also do so when they encounter certain asteroids and aeolids, moving away afterwards by repeated flexions of the body (Yentsch and Pierce, 1955; Sund, 1958; Robson, 1961). These activities have provided opportunities for studying the behavioral physiology of actinians in general and have contributed to the discovery of the conduction systems that control behavior in these animals (McFarlane, 1969a,b; Lawn, 1976).

The present study began with the observation that specimens believed to belong to *Tealia crassicornis* released their pedal disks when they came into contact with the leatherstar, *Dermasterias imbricata.* Attempts to confirm this gave variable results, a difficulty that was only removed later when Sebens and Laakso (1977) showed that some anemones previously assigned to *T. crassicornis* ranked as a separate species which they named *T. piscivora.* When we tested both species separately we found that specimens of *T. crassicornis* never released their pedal

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disks in response to *D. imbricata* whereas specimens of *T. piscivora* usually did so. About the same time specimens of an undescribed north Pacific *Tealia* were collected which gave an even more striking releasing response to *D. imbricata.* The behavioral physiology of this animal is the subject of this paper. Pending a full systematic description we shall name it *Tealia* sp. A laboratory and subtidal study is now in progress on the behavior and general ecology of *T. piscivora.* This will be the subject of a separate paper.

## MATERIALS AND METHODS

*Tealia* sp. was collected in Barkley Sound, British Columbia, at depths of 75–110 m. Exact locations and depths are not known because all 8 specimens obtained during 1978–80 came up on separate occasions in a fisherman's net. Another specimen was found in the display tank at the Friday Harbor Laboratory in 1981, but the site and time of its collection are not known. We present here a few features of the 8 animals in our collections: diameter of the tentacular crown, 5–20 cm, and of the pedal disk, 3–14 cm; height of column 4–20 cm; color of column, translucent, grading through pale mauve to pink below the margin; tiny beadlike verrucae on the column in irregular horizontal rows. The animal was judged to be a species of *Tealia* from the decamerous arrangement of the innermost ring of tentacles, the presence of a fosse, the long stout tentacles, and the presence of verrucae resembling those of some other species of *Tealia.* It did not correspond to any known species of *Tealia* as described by Stephenson (1935), Carlgren (1949), and Hand (1955). With so few animals available, priority was given to behavioral and physiological work on the living anemones before preserving specimens for identification.

The animals were kept in aquaria at the Bamfield Marine Station and were fed about once per week on pieces of fish or mollusks. *D. imbricata* and other asteroids were presented to individual anemones and their responses noted and timed. Typical responses were recorded in still and motion pictures for further study.

Electrophysiological techniques followed the standard procedure developed for sea anemones (McFarlane, 1969a,b; Lawn, 1976, 1980). A polyethylene suction electrode was attached to a tentacle for recording purposes, and a similar stimulating electrode was attached to the column.

## RESULTS

### *Behavioral observations*

The behavior of *Tealia* sp. and five described species of *Tealia* (*T. coriacea, T. crassicornis, T. lofotensis, T. columbiana, T. piscivora*) was first studied in 10 presentation trials with *Dermasterias imbricata.* The sea stars were brought into contact with firmly attached anemones and kept in contact for 3 min or until the anemone detached its pedal disk. *Tealia* sp. released its pedal disk in 9 and *T. piscivora* in 8 of the 10 trials. None of the other species ever responded in this way. The tentacles of species that did not release normally clung strongly to the sea star as to food, whereas *Tealia* sp. and *T. piscivora* remained in contact without clinging.

Specimens of 14 other asteroids were available at Bamfield for trials with *Tealia* sp. similar to those described above with *Dermasterias.* Only one of these species, *Patiria miniata*, caused frequent release of the pedal disk, 7 times in 20 trials. Two other species caused the pedal disk to release occasionally: *Solaster stimpsoni* once in 20 trials; *Crossaster papposus* once in 14 trials. The following 11 species, each tested 20 times, never caused the pedal disk to release: *Evasterias troscheli, Henricia leviscula, Hippasteria spinosa, Leptasterias hexactis, Mediaster aequalis, Orthasterias koehleri, Pisaster brevispinus, Pisaster ochraceus, Pteraster tesselatus, Pycnopodia helianthoides, and Solaster dawsoni.*

Release times provided further evidence that *Dermasterias* (mean time 33 sec in 14 releases) was considerably more effective than *Patiria* (mean time 78 sec in 7 releases). The single releases to *Crossaster papposus* and *Solaster stimpsoni* took place at 130 and 180 sec, respectively. These results suggest that the release is triggered by substances which are present and deliverable in amounts that can cause release frequently in only two of the asteroids tested. Possibly these substances occur in other asteroids also but only at levels that are usually below the threshold that causes the release of the pedal disk.

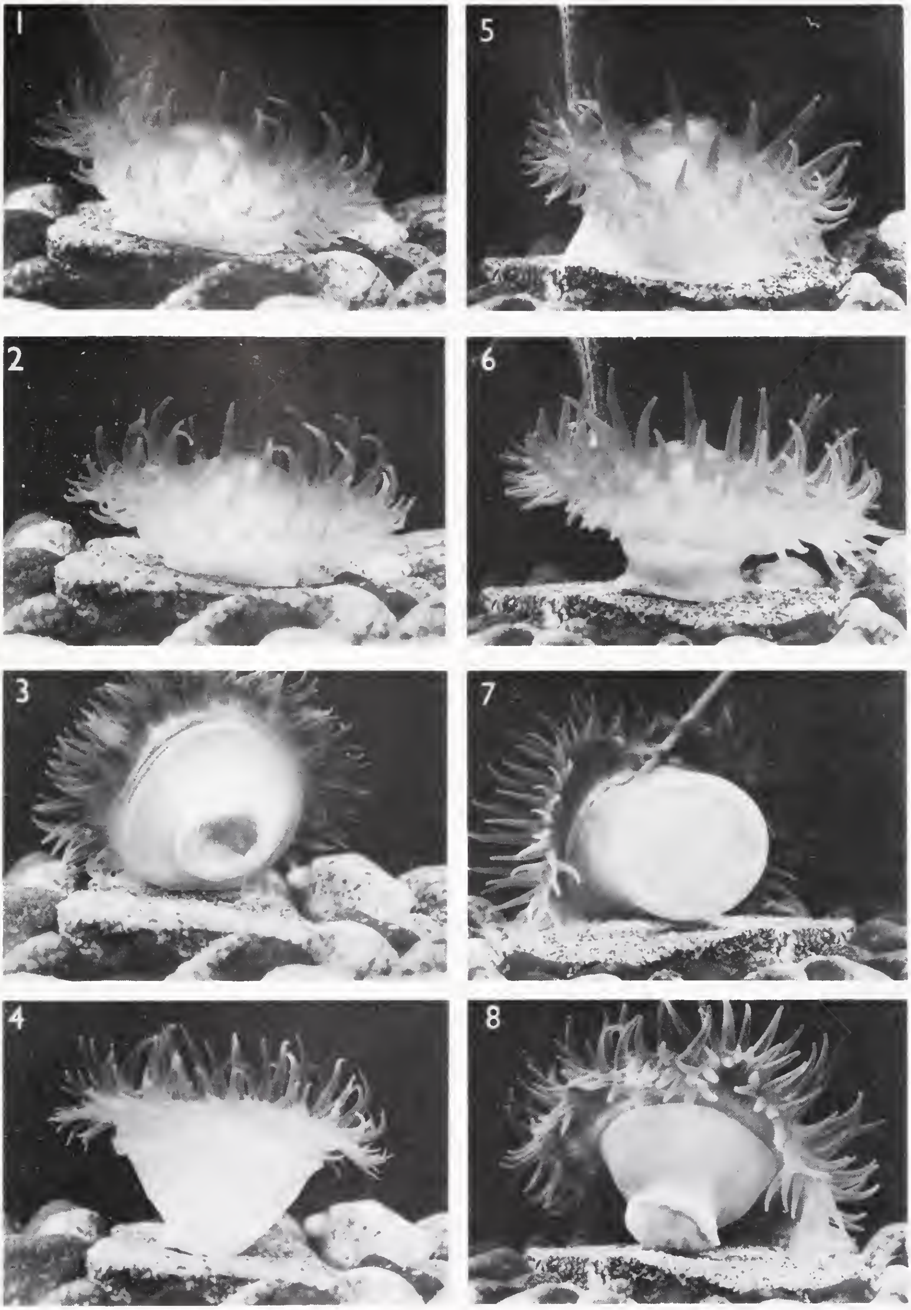
Unlike the responses of *Stomphia* spp. to *Dermasterias*, etc., the release of the pedal disk in *Tealia* sp. is not accompanied or followed by asymmetrical flexions ("swimming") or other repetitive activity. Resettlement often followed quickly, within 2–5 min, if the anemone remained upright. When the anemone fell over or was carried away by a current, resettlement did not begin until the pedal disk came against a surface to which it could adhere.

One of the 8 specimens of *Tealia* sp. was much larger than the others (pedal disk diameter 14 cm). This animal failed to release in response to *Dermasterias* on a number of occasions; in fact most of the trials which failed to cause release occurred with this animal. We found that the small and medium-sized specimens of *Tealia* sp. in our small collection gave more consistent and more rapid responses, and they were used more frequently in our tests. If a larger supply of animals could become available it would be interesting to see if a relationship exists between the size of the anemone and the frequency and speed of the release.

Figures 1–4 show a typical release of *Tealia* sp. when its tentacles were touched by *D. imbricata.* On a moderately extended specimen with the sea star touching the tentacles (Fig. 1) the diameter of the tentacular crown increased, the oral disk became convex, and the column shortened dramatically to about one-quarter of its original length (Fig. 2). These changes took place by slow, smooth movements, almost imperceptible as they happened but transforming the animal completely in less than half a minute.

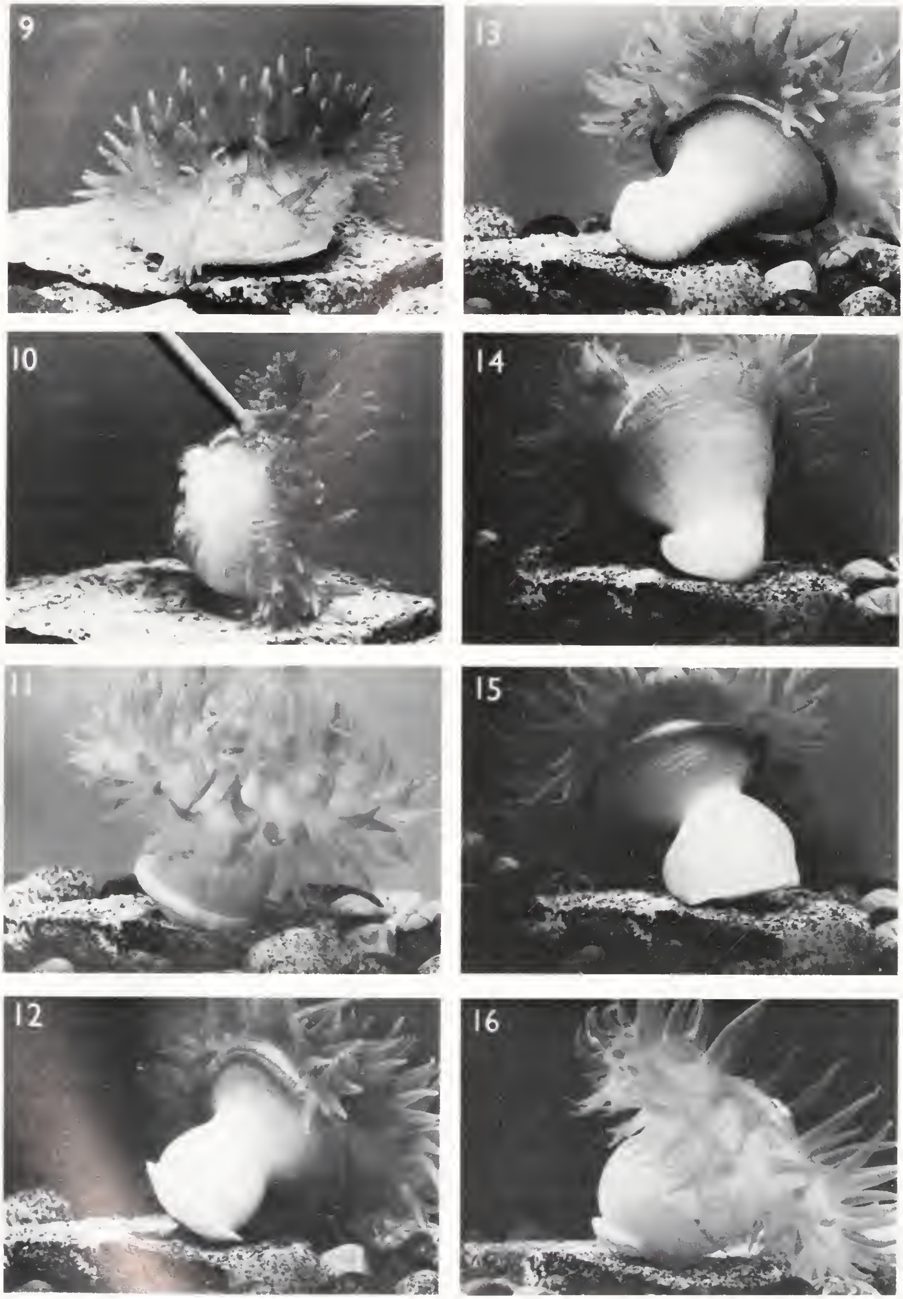
The shortening of the column was due in part to a constriction of the margin of the pedal disk to form a tightly contracted ring. Consequently the pedal disk no longer adhered and the anemone became detached and took on the shape of an inverted, almost medusoid, cone with the tentacular crown flared out and the pedal disk deeply concave (Fig. 3). Later, as the anemone began to resettle, about 3 min from the beginning, the column extended, and the constricted pedal disk began to adhere and extend outwards to bring the animal back to its normal position and appearance (Fig. 4).

Figures 5–8 show another example. This animal had a short column at the beginning (Fig. 5). About 15 sec after *Dermasterias* was brought in, the pedal disk lifted following the narrowing of the base (Fig. 6) and the flaring out of the crown and oral disk. Side views show the detached and almost flat pedal disk with a pattern of concentric grooves and at the center a slight elevation with a small central pit (Fig. 7). Later the base narrowed and resembled a terminal knob with the upper margin and crown still flared out and completely inactive, giving the animal the shape of a flower vase (Fig. 8). Then the pedal disk slowly assumed its normal dimensions and settlement proceeded as the disk spread across the stone. The entire response took less than 10 min.



FIGURES 1–8. *Tealia* sp. Two examples of pedal disk release and subsequent changes in shape in response to contact with *Dermasterias imbricata*. Full description in text. Time elapsed between Figures 1 and 4 approx. 2.5 min and between Figures 5 and 8 approx. 3.0 min.

There were minor variations around these patterns in the responses of our animals. Examples of some of the shapes assumed at various times are shown in Figures 9–16. It often happened also that an animal seemingly about to resettle



FIGURES 9–16. *Tealia* sp. Characteristic postures after release of pedal disk in response to *Dermasterias*. Note release and shortening of column (9, 10), swelling of pedal disk with upturned basal margin (11, 12, 13), extension of column with intermittent peristaltic waves (12, 14, 15, 16).

would fail to do so and would assume strange shapes and postures with strong peristaltic waves passing orally, before finally settling down.

The nudibranch, *Aeolidia papillosa*, which can cause *Stomphia* to detach and swim, induced detachment in *Tealia* sp. in three out of 11 trials. The release times

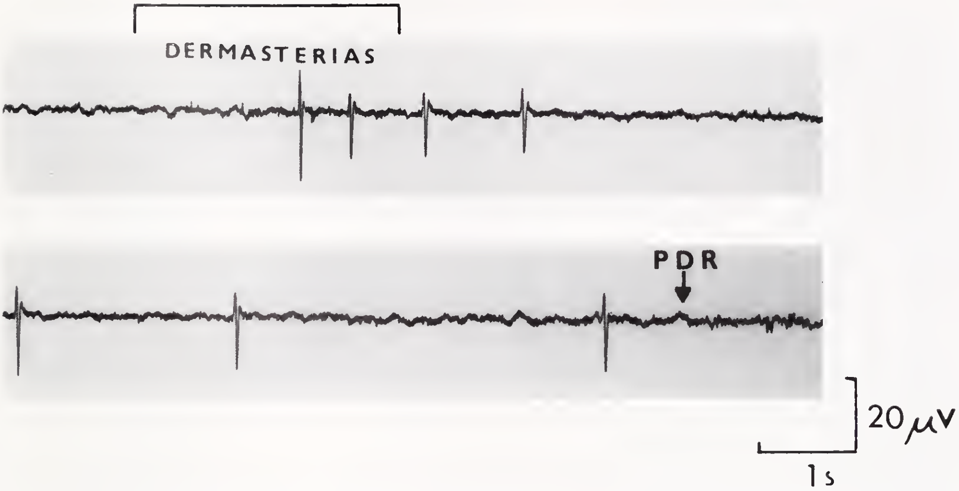


FIGURE 17. Electrical activity recorded from tentacles of *Tealia* sp. during and following 2.8 sec contact between *Dermasterias* and tentacles of anemone. Note 7 typical SS1 pulses. Arrow at PDR marks time of release of pedal disk. (Continuous trace; duration approx. 17 sec).

were long, 75, 80 and 240 sec. The responses differed from the response to the asteroids. Instead of spreading the oral disk outward, the anemone closed up and then released its pedal disk. It is interesting to note that *Hippasteria spinosa*, an asteroid that is highly successful in causing *Stomphia* to detach and swim, had no obvious effect on *Tealia* sp.

### *Electrophysiological data*

McFarlane (1969a) and Lawn (1976) have described three types of pulses that have been recorded from sea anemones using standard electrophysiological techniques. These pulses are attributed to three different conduction systems that have become known by their initials: 1) NN (through-conducting nerve net located in the endoderm); 2) SS1 (slow system 1 located in the ectoderm); 3) SS2 (slow system 2 located in the endoderm). Each system has been linked to specific behavior patterns, *e.g.* NN with retraction and closure. SS1 pulses coincide with the release of the pedal disk in two cases: in *Calliactis parasitica* SS1 pulses accompany the slow release of the pedal disk in transfers to shells from other surfaces (McFarlane 1969b, 1973); in *Stomphia coccinea* SS1 pulses precede the release of the pedal disk in encounters with *Dermasterias* (Lawn, 1976). In a European *Tealia, T. felina* var. *lofotensis* (nomenclature of Stephenson, 1935), SS1 pulses triggered the prefeeding response that occurs when food substances come into contact with the column (McFarlane, 1970; Lawn, 1975). Thus there was reason to believe that SS1 pulses would be recorded in *Tealia* sp. and special interest attached to finding out whether SS1 pulses were associated also in this animal with the release of the pedal disk.

Figure 17 shows a typical record of the electrical activity in *Tealia* sp. when *Dermasterias imbricata* was brought up to the tentacles for a few seconds. Two SS1 pulses of characteristic appearance occurred about 2 sec after contact. Two more pulses followed at 1 sec intervals and three more followed with the intervals between pulses becoming longer until the anemone released its pedal disk, about 14 sec after the sea star made contact. This strongly suggests that the detachment of the pedal disk in *Tealia* sp. is triggered by a series of SS1 pulses.

The record shown in Figure 17 was preceded by a trace lasting several minutes from which SS1 pulses were conspicuously absent. Similar records were obtained, with pretrial controls, from three other specimens of *Tealia* sp. Further trials were carried out which gave similar records when the anemones had resettled. The relationship between SS1 pulses and the release of the pedal disk as seen in Figure 17 is not a statistical event; in our experience the two invariably occur together.

The SS1 pulses recorded before the detachment of *Tealia* sp. are almost identical with those that precede the detachment of the swimming anemone *Stomphia* (Lawn, 1976). The time that elapses between the application of the stimulus, the sea star, and the beginning of the response, is of the same order, usually a few seconds. The number of SS1 pulses preceding detachment is also of the same order in the two cases, usually from 6 to 12.

Examining records from a number of interactions between *D. imbricata* and *Tealia* sp. shows a good deal of variation in the number and the timing of pulses that trigger the release of the pedal disk on different occasions. A recurrent feature of the records was a tendency for two or three pulses to occur close together soon after contact was established between the two animals.

*Pateria miniata*, as reported earlier, sometimes evoked the releasing behavior, and it also set up trains of SS1 pulses. Of 10 trials, four resulted in detachment. The pulses in these records were similar to those in the interactions with *Dermasterias*, though generally the firing rate was lower, and in cases where release did not occur the pulses ceased after an initial two or three. Maintaining the activity after these first few pulses seems to be important to the triggering function.

Electrical stimulation of the SS1 in *Tealia* sp. caused detachment of the pedal disk and inflation of the oral disk. It was not possible to cut ectodermal flaps successfully in this anemone (McFarlane, 1969b), and this meant that the SS1 could not be stimulated separately from the nerve net. Results showed that the effective frequencies of stimulation fell in the range of one shock every 3 sec to one shock every 10 sec. The minimum number of shocks required to produce a response varied from four to eight depending on frequency. This corresponds closely to the situation previously encountered in *Stomphia* (Lawn, 1976).

## Discussion

These results give rise to discussion on three topics: 1) comparative aspects; 2) the activity and mobility of the pedal disk; 3) the adaptational significance of the releasing behavior in *Tealia* sp.

### *Comparative aspects*

Detachment in response to specific stimuli seems to be a general adaptation in a few genera in certain families. *Boloceroides*, in the family Boloceroididae, is a lightly attached Indo-Pacific anemone that releases quickly and swims actively in response to a nudibranch predator (Lawn and Ross, 1982). *Stomphia* spp. in the large family Actinostolidae, few of whose species have been observed alive, release quickly and "swim" in response to certain sea stars and nudibranchs (Robson, 1966; Ross, 1974). *Calliactis* spp., and other symbiotic actinians in the family Hormathiidae that live on crustaceans and gastropods, release their pedal disks slowly in response to shells or to the manipulations of certain hermit and spider crabs (Ross, 1974).

*Tealia* belongs to the Actiniidae, most of which are very firmly attached and difficult to dislodge, *e.g. Actinia* spp. This description applies to the familiar northeast Pacific species, *T. coriacea* and *T. crassicornis*. The fact that *Tealia* sp. and

*T. piscivora* can release quickly in interactions with certain animals in their environments, whereas four other species of *Tealia* cannot do so, shows that behavioral attributes often differ within taxa of generic or higher rank; within a genus or family these attributes may be restricted to particular species only. We see in this another example of the versatility of neuromuscular mechanisms in the actinians, often without any external signs of such special adaptations.

It is instructive to compare the behavioral physiology of *Tealia* sp. and *Stomphia* spp. in releasing the pedal disk. The SS1 conduction system is activated in both cases by the same stimulus, *Dermasterias*. The SS1 pulses trigger the release in similar ways; the number of pulses required, their frequency, and the latency of the response are of the same order of magnitude in both animals. However, the behavioral events that accompany and follow the release differ. In *Tealia* sp. the column shortens and the oral disk and tentacles flare out into an immense corona and contain most of the coelentric fluid. After this transformation, the anemone slowly returns to normal and resettles. The entire behavior proceeds without any movements except slow symmetrical changes of shape. Moreover, there is no evidence of the post-release or post-swimming torpor of *Stomphia* spp. Once *Tealia* sp. releases, the tentacles become extremely adhesive, unlike those of *Stomphia* which are non-adherent at this time. Whereas in *Tealia* sp., the column is greatly shortened during the release, in *Stomphia* it is greatly extended, and the release is accompanied and followed by swimming flexions for 2–3 min. Thus, almost identical triggering systems are used in the two cases to achieve different ends.

### *The pedal disk*

These results reemphasize the sensory and motor activity taking place in the pedal disk in these special behavior patterns. Earlier examples were: the demonstration by Davenport *et al.* (1961) that the clinging of the tentacles of *Calliactis* on shells depended on information as to whether the pedal disk was on a shell or not; the description of the release and the resettlement of *Calliactis* and *Stomphia* showing the pedal disk to be an area in which many activities take place, *e.g.*, the symmetrical constriction bringing about release in *Calliactis* (Ross and Sutton, 1961); the swelling of the pedal disk to make contact with surfaces for settling in *Stomphia* and *Paracalliactis* (Ross, 1974); the description of the asymmetrical locomotory movements in *Metridium* (Batham and Pantin, 1950). The behavior of *Tealia* sp. described above provides another example of the activity of the pedal disk in actinian behavior, especially in anemones that abandon their sedentary habits from time to time.

### *Adaptational significance*

The adaptational significance of the detachment behavior of *Tealia* sp. is far from clear. By analogy it looks like an escape response but there is no evidence that asteroids prey on *Tealia* sp. If it is an escape reaction, the anemone may employ it to escape from some other predator not yet discovered. Once detached, the expanded *Tealia* sp. is virtually weightless so that any current would carry it away and remove it from a potential predator. However, questions about the adaptational significance of the releasing behavior in *Tealia* sp. can only be answered with data from subtidal observations. Such studies are now in progress with *T. piscivora*. Unfortunately, *Tealia* sp. has been collected infrequently, its normal habitat is unknown, and it has not yet been located by divers, so we have no immediate prospect of observing it in nature.

## ACKNOWLEDGMENTS

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# PUMPING RATES AND PARTICLE RETENTION EFFICIENCIES OF THE LARVAL LAMPREY, AN UNUSUAL SUSPENSION FEEDER

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## ABSTRACT

The suspension feeding larvae of lampreys (ammocoetes) inhabit fine-grained sediments where particulate organic matter is concentrated, but whose low permeability limits the rate at which ammocoetes can pump water (flow rate). This study determined: 1) flow rates through the pharynges of ammocoetes, both within and out of the sediment, and 2) the ability of ammocoetes to filter particles from suspension (retention efficiency) over a wide range of algal cell concentrations (*Chlorella pyrenoidosa*, 1–75 mg/l).

For most suspension feeders, flow rate and retention efficiency must be measured indirectly (clearance method). Direct measurement was possible here, as ammocoetes remain apparently undisturbed in glass tubes that allow the separation of inhalent from exhalent ventilatory currents. Problems arise in attempting to use clearance methods to determine flow rates in burrowed suspension feeders, and these problems are discussed.

Ammocoete flow rates are exceptionally low compared to the rates of other suspension feeders, but retention efficiency was consistently high, even at the highest algal concentrations employed ($\bar{x} = 82\%$). While most suspension feeders rapidly process dilute suspensions, ammocoetes meet nutrient needs by slowly processing concentrated suspensions.

## INTRODUCTION

Lampreys spend most of their life cycle as suspension feeding larvae (ammocoetes), living within the sediment of stream beds (Hardisty and Potter, 1971; Potter, 1980). Ammocoetes occupy burrows that are either open at one end (mouth) or are fully closed off from the overlying water. Suspended food particles are obtained from the water just above the substrate surface, and also from pore water within the sediment (Moore and Mallatt, 1980). Feeding involves trapping small particulate detritus and unicellular algae on mucus within the pharynx (Mallatt, 1979, 1981). Water is propelled by rhythmic muscular contractions of the pharyngeal wall, and by a pair of muscular flaps, the velum, at the anterior end of the pharynx (Rovainen and Schieber, 1975). Observations in this laboratory indicate ammocoetes extrude their exhalent water into the substrate around the burrow. Since the sediments occupied are fine sands and muds (see Fig. 1, and Malmqvist, 1980) of low permeability, ammocoetes must pump water against resistance. The thick, particle-trapping mucus, which fills most of the pharynx (Mallatt, 1981), also is likely to impede water flow.

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Abbreviations: F, rate of water flow through pharynx; F′, clearance rate; RE, retention efficiency; W, wet weight of larvae.

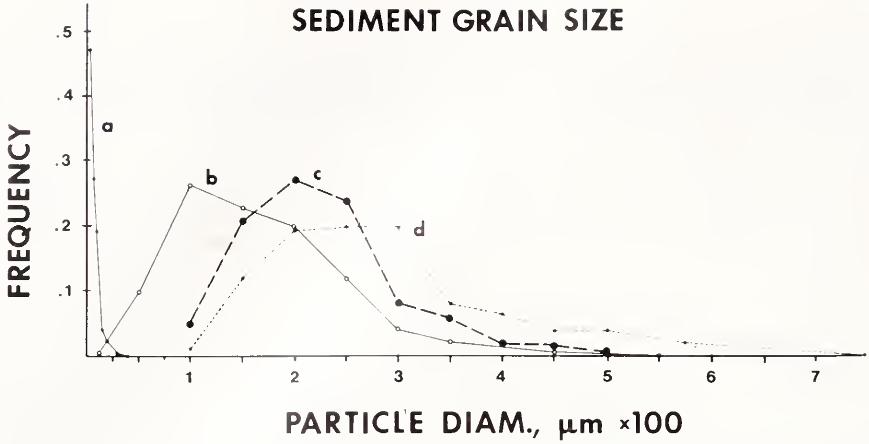

FIGURE 1. Size-frequency distributions of particles comprising several sediments in which ammocoetes will burrow and feed. That labelled 'a' is a diatomaceous earth, 'b' and 'c' are commercially obtained 70-mesh silica sands, and 'd' is a sand from an ammocoete habitat (*Petromyzon marinus*, Pere Marquette River, Michigan). Sand 'c' was used throughout this study. The numbers of grains measured exceeded 175 in all cases. As a measure of permeabilities, the times taken to drain 10 cm of water through 10 cm sediment columns are: a: 21 min; b: 6.5 min; c: 2 min; d: 2.75 min.

A search of the literature on suspension feeding animals (see especially Jørgensen, 1966, and Wallace and Merritt, 1980) revealed no other instance in which water is characteristically propelled into a fine-grained substrate. Most suspension feeders are either pelagic (crustacean zooplankton: Jørgensen, 1975; rotifers: Starkweather, 1980; frog tadpoles: Seale *et al.*, 1982), or if benthic, are epifaunal (bivalves: Winter, 1973; ascidian tunicates: Randløv and Riisgård, 1979). The infaunal suspension feeders that have been studied either inhabit coarse permeable sediment (amphioxus: Azariah, 1969; Webb, 1975) or have full access to the overlying water. Such access is achieved through U-shaped burrows (mayfly and midge larvae), siphons with inhalent and exhalent openings (infaunal bivalves), or protruding the filter into the overlying water (many polychaetes). In pumping against resistance, ammocoetes are unusual among suspension feeders. In fact, the existence of factors threatening to limit the rate at which ammocoetes can pump water seems to clash with the basic tenet of suspension feeding that large quantities of water must be processed rapidly (Jørgensen, 1975). How does such a suspension feeder survive?

Another unusual feature of ammocoete ecology is that the habitat contains comparatively high concentrations of suspended food particles. Due to natural processes of particle settling and resuspension, suspensions are expected to be more concentrated at the floor of a natural body of water than in the water column above (Hardisty and Potter, 1971). Supporting this, Moore (Moore and Potter, 1976, Fig. 1b; Moore and Mallatt, 1980, Fig. 1) measured higher levels of suspended organic solids at the substrate in ammocoete habitats (1–40 mg/l) than typically are present in open waters, where many other suspension feeders are found (below 1 mg/l; Jørgensen, 1975). The nature of their habitat suggests ammocoetes can efficiently process concentrated suspensions, and this merits experimental investigation.

With the special ecological features in mind, this work determines the rates at which ammocoetes pump water (flow rate) when in and out of sediment, and the efficiency with which they remove food particles (retention efficiency) from suspensions of different concentrations.

Larval lampreys are ideal experimental animals for this type of study. For most other suspension feeders, flow rate and retention efficiency must be estimated indirectly, through monitoring the rates at which they clear particles from the water (clearance rates: see Jørgensen, 1975, for discussion). To utilize such a method, however, one must assume at some point that retention efficiency is 100%, an assumption that is untestable for most animals. For ammocoetes, flow rate and retention efficiency can be measured directly, as the larvae will feed in tubes, which allow separation of inhalent and exhalent currents.

This study provides some kinds of data seldom obtained for suspension feeders. Flow rates are measured in the absence of food particles, not possible with indirect methods. Also, this is one of the first studies in which retention efficiency is investigated as a function of particle concentration (also see Kurtak, 1978). In most past studies that have employed direct techniques on suspension feeders (Fiala-Médioni, 1978; Randløv and Riisgård, 1979), retention efficiency was related only to particle size.

Data on the flow rates of ammocoetes in glass tubes are supplemented by clearance rate data from burrowed individuals. Special problems arise in attempting to use indirect methods to determine the flow rates of burrowing suspension feeders *in situ*, and these are documented here.

## MATERIALS AND METHODS

This study primarily utilized larval *Petromyzon marinus*, which were obtained from the Muskegon and Pere Marquette rivers, Michigan, and from the Hammond Bay Biological Station, Millersburg, Michigan. A few Pacific lamprey ammocoetes (*Lampetra tridentatus*) were used, obtained from the Potlatch River near Bovill, Idaho. (Ammocoetes of different species are quite similar, morphologically and physiologically: Hardisty and Potter, 1971.) Stock animals fed on yeast and grew normally, averaging a 10% weight increase per month (Mallatt, unpublished). Experimental animals were between 10.1 and 11.1 cm long, with wet weights between 1.3 and 2.0 g ($\bar{x} = 1.6$). All experiments were performed at 12°C.

The use of glass tubes to measure flow rate was inspired by Rovainen and Schieber (1975). Test chambers were glass pans (Fig. 2) divided into anterior and posterior compartments, holding 150 and 1100 ml of water, respectively. The water was dechlorinated tap water, previously filtered through a 0.45 $\mu$m Millipore® filter; water was continuously aerated in both compartments. The test animal occupied a glass tube, which pierced the partition. *P. marinus* larvae were employed whose pharynges fit snugly but without constriction into the tubes (0.6 cm internal diameter). Flow rates were monitored in dim light. Water pumped by the animal from the anterior compartment was replaced continuously, and the flow rate was considered to be the replacement rate (ml/hr, later adjusted for animal mass). Differences in water height between anterior and posterior chambers were kept low (<0.5 cm). It was determined, through removal and addition of known amounts of water, that the mean and maximum errors in the measure of flow rate were ±2 and ±5 ml/hr, respectively. In preliminary tests, dye (Methyl blue) added to the posterior compartment did not color water in the anterior compartment over an eight hour period with the ammocoete in place, so flow was unidirectional as expected.

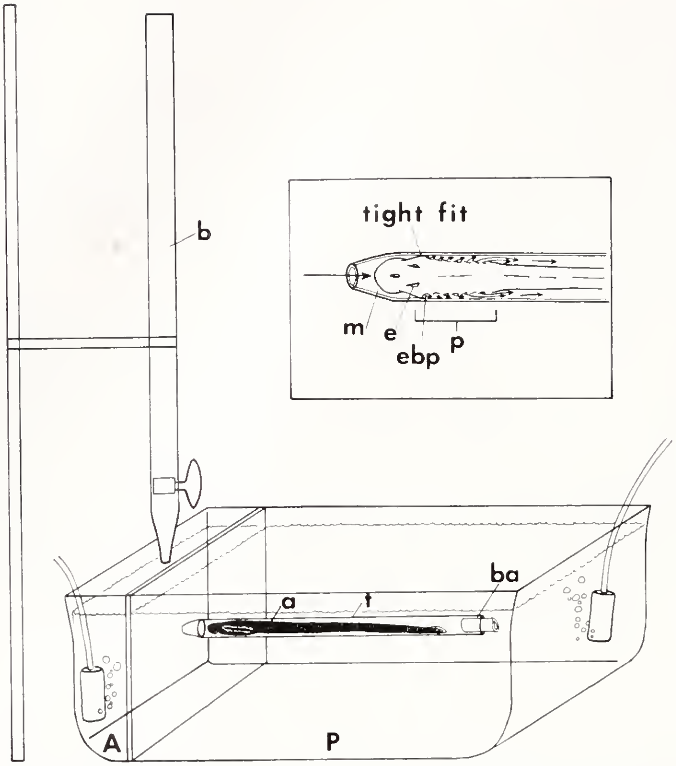

FIGURE 2. Apparatus employed for measuring: 1) flow rate through the pharynx, and 2) particle retention efficiency, of larval *P. marinus*. Ammocoete (a) in the tube (t) pumps water from the anterior compartment (A) to the posterior compartment (P). Inset shows tube in dorsal view. Other symbols: b, buret; ba, balloon attachment site; e, eye; ebp, external branchiopores; m, mouth; p, pharynx.

The experimental procedure involved monitoring the flow rates of 12 individual ammocoetes for periods of 4 to 13 hours, after an 18 hour period of adjustment to the apparatus. The reason flows were monitored over time was to determine whether the confinement of the tubes stressed the ammocoetes, as might be reflected in a cumulative tendency to increase or decrease flow rate (Cairns *et al.*, 1982).

For measuring the clearance rates of buried ammocoetes, an indirect technique was used, similar to that of Malmqvist and Brönmark (1982). The test chambers were five-liter aquaria, containing four liters of continuously aerated, dechlorinated tap water, one liter of which occupied the interstices in two liters of a silica sand (Fig. 1, sand 'c'). Two test aquaria were employed, one containing *P. marinus*, and the other, *L. tridentatus* ammocoetes (four to six per tank, 8–12 g). Each test tank was paired with a control tank that lacked ammocoetes.

This test of clearance rates lasted two months, with trials conducted daily. At the onset of each trial, fresh yeast suspension (*Saccharomyces cerevisiae*, Fleischmann's® cakes) was added to the water above the sediment of test and control tanks, in amounts that varied randomly from trial to trial, to yield particle concentrations ranging from 5 to 2700 mg (dry) per liter. Yeast cell concentrations in the water above the sand (C) were measured visually with a hemocytometer at the onset ($C_1$) and the end ($C_2$) of the four to eight hour duration (T) of each trial. The rates at which burrowed ammocoetes cleared particles from the overlying water ($F'$, ml/g/h) were calculated according to the equation:

$$\text{Clearance Rate} = F' = \frac{3000\text{ ml}\,[(\ln C_{1,t} - \ln C_{2,t}) - (\ln C_{1,c} - \ln C_{2,c})]}{T \cdot W}$$

where 3000 ml is the volume of overlying water, W is the wet weight of the larvae in the test tank, and the subscripts t and c denote test and control tanks, respectively (Coughlan, 1969). The water was changed and the sand was washed after each trial. Trials were conducted in the dark. Variation in cell settling rates in control *versus* test tanks led to the occasional calculation of negative clearance values; these were treated as zero (or as one ml/g/h for log-transformed data).

Another experiment was performed to relate flow rates measured for ammocoetes in tubes to the clearance rates of burrowed individuals. Here, the effect of the sand's resistance on flow rate was tested directly. Three *P. marinus* larvae that had been used in clearance studies were placed in the tube devices. A flexible plastic tube was then fit snugly around the posterior end of each glass tube, and the rate at which the ammocoete propelled water through the plastic tubing was monitored before and after the insertion of a plug of sand. The latter was 4 cm long, approximating the depth at which many buried ammocoetes resided in stock tanks. The plastic tube was bent slightly to assure the sand entirely filled the width of its lumen.

Several things should be noted about the construction of Table I, where flow rates are compared among many groups of suspension feeders. These figures can

TABLE I

*Flow rate in different suspension feeders*

| Animal | Wet weight (g) | Flow rate (ml/g/h) | Flow rate adjusted to 1.6 g wet body mass[a] |
|---|---|---|---|
| 1. Copepods | | | |
| a. *Calanus helgolandicus* | $1.2 \times 10^{-3}$ | 15,800 | 2,600 |
| b. *Calanus pacificus* | $1 \times 10^{-3}$ | 7,600 | 1,200 |
| 2. Lamellibranches | | | |
| a. Various bivalves[b] (13 species) | 1.6 | 600–5000<br>$\bar{x} = 1600$ | 600–5000<br>$\bar{x} = 1600$ |
| b. *Crassostrea virginica* | 1.0 | 1,580 | 1,560 |
| c. *Pecten irradians* | 3 | 1,000 | 1,170 |
| d. *Mytilus edulis* | 1.6 | 190–625 | 190–625 |
| e. Various bivalves[b] (3 species) | 1.6 | 125–625 | 125–625 |
| f. *Dreissena polymorpha* | $5 \times 10^{-1}$ | 150 | 110 |
| 3. Cladocerans | | | |
| a. *Daphnia pulex* | $2.2 \times 10^{-5}$ | 3,400 | 370 |
| b. *Daphnia magna* | $5.0 \times 10^{-5}$ | 4,000 | 530 |
| 4. Rotifers | | | |
| a. *Keratella cochlearis* (large) | $3.7 \times 10^{-7}$[c] | 21,600 | 470 |
| b. *Keratella cochlearis* (small) | $1 \times 10^{-7}$ | 25,000 | 400 |
| c. *Conochilus dossuarius* | $6 \times 10^{-7}$ | 9,700 | 240 |
| d. *Kellicottia bostoniensis* | $3.7 \times 10^{-7}$ | 2,300 | 50 |
| 5. Sponges | | | |
| a. *Sycon coronatum* | 1.25 | 980 | 920 |
| b. *Halichondria panica* | 3.0 | 370 | 430 |

TABLE I—(*Continued*)

| Animal | Wet weight (g) | Flow rate (ml/g/h) | Flow rate adjusted to 1.6 g wet body mass[a] |
|---|---|---|---|
| 6. Bryozoan | | | |
| *Zoobotryon verticillatum* | $5.5 \times 10^{-5}$ | 6,700 | 290 |
| 7. Ciliates | | | |
| a. Algavores (large cells) *e.g.*, *Stylonychia mytilius* | $5 \times 10^{-8}$ | 500,000 | 1,300 |
| b. Feeders on intermediate-sized cells (2–5 μm diam) *e.g.*, *Paramecium* | $2.5 \times 10^{-8}$ | 24,000 | 270 |
| c. Bacterivores *e.g.*, *Tetrahymena pyriformes* | $1 \times 10^{-8}$ | 5,000 | 45 |
| 8. Infaunal Polychaetes, and other burrowing worms | | | |
| a. Sabellidae | | | |
| *Myxicola infundibulum* | 2.7 | 100 | 115 |
| *Schizobranchia insignis* | 1.0 | 70 | 60 |
| *Sabella pavonina* | $1.9 \times 10^{-1}$ | 390 | 230 |
| b. Serpulidae | | | |
| *Potamoceras triqueter* | $1.9 \times 10^{-2}$ | 1,400 | 460 |
| *Hydroides norvegica* | $1.2 \times 10^{-2}$ | 900 | 260 |
| *Spirorbis borealis* | $2 \times 10^{-4}$ | 950 | 100 |
| *Salmacina dysteri* | $1 \times 10^{-4}$ | 2,090 | 190 |
| c. *Chaetopterus variopedantus* | 6 | 50 | 70 |
| d. *Urechis caupo* (Echiuroidea) | 21 | 900 | 1,230 |
| 9. Chordates | | | |
| a. Various tunicates (7 genera) | 1.6 | 95–560, $\bar{x} = 225$ | 95–560, $\bar{x} = 225$ |
| b. *Branchiostoma lanceolatum* | $1.5 \times 10^{-2}$ | 200–316 | 70–100 |
| c. *Hyla crucifer* (frog tadpole) | 0.2 | 25–65 | 15–40 |
| d. *Bufo woodhousei* (frog tadpole) | 0.15 | 50–140 | 30–80 |
| e. Larval *Petromyzon marinus* | 1.6 | | 8–64, $\bar{x} = 28$ (in tube) |

SOURCES: 1a. Paffenhöfer (1976), Fig. 3; 1b. Runge (1980), Table 3 (September value); 2a. Møhlenberg and Riisgård (1979); 2b. Palmer (1980), Table III; 2c. Jørgensen (1966); Fig. 1.40; 2d,e. Foster-Smith (1975), Figs. 1,2; 2f. Walz, (1978), Fig. 2; 3a. Crowley (1973); 3b. Ryther (1954), Figs. 2,4; 4. Bogdan *et al.* (1980), p. 74–75; 5a,b. Foster-Smith (1976), Table IV; 6. Bullivant (1968); 7. Fenchel (1980a), p. 18 and Fig. 4; 8. Jørgensen (1966), Table 1.1 and p. 11; 9a. Randløv and Riisgård (1979), Fig. 4; 9b. Azariah (1969); 9c,d. Seale and Beckvar (1980); 9e. present study.

NOTE: Essential data on food type and experimental temperature are as follows: 1a. Various algae at a range of concentrations, 15°C; 2a. Various unicellular algae, 2 to 10 × $10^4$ cells/ml, 10–13°C; 2b. *Thalassiosira, Isochrysis* and *Dunaliella*, 21°C; 2c. "Flagellates and diatoms", 22–26°C; 2d,e. No information given; 2f. *Nitschia actinastroides*, 0–24 mg/l, 15°C; 3a,b. *Rhodotorula* sp., 20°C; 4a,c,d. *Chlamydomonas*, 20°C; 4b. *Rhodotorula*. 20°C; 5a,b. No information given; 6. *Monochrysis*, 24°C; 7. See legend to Fenchel's (1980a) Fig. 4; 8a–c. Colloidal graphite particles, 16–20°C; 8d. Direct measurement, 20°C; 9a. See legend to Fig. 4 in Randløv and Riisgård (1979); 9b. "Normal sea water", 29°C; 9c,d. *Anabaena sphaerica*, 0.2–20 mg/l, 21°C; 9e. Unfed, or fed on *Saccharomyces cerevisiae*, 12.5°C.

[a] Assumes $F \propto W^{0.75}$: See Materials and Methods section. Values also assume animals' dry weights are 20% of wet weight.

[b] See Winter (1973, 1978) and Foster-Smith (1976) for more data on flow rates in bivalves.

[c] Rotifer weights calculated from body lengths assuming $W \propto L^3$ between genera.

be compared only broadly, as measurements reported in the literature were obtained by different methods and at different temperatures (but mostly between 12 and 22°C). An attempt was made to correct for the most important source of variation in published values of flow per body mass (F/W), the effect of animal size. This was done through assuming $F \alpha W^{0.75}$, *i.e.*, $F/W \alpha W^{-0.25}$. Studies on a variety of suspension feeders support this assumption (Azariah, 1969; Paffenhöfer, 1971; Jørgensen, 1975; Møhlenberg and Riisgård, 1979; Fenchel, 1980a; Palmer, 1980), and Winter (1978) discussed it at length. In constructing the table, it was also necessary to assume dry weights of the animals were 20% of wet weight.

Retention of *Chlorella pyrenoidosa* cells was measured for *P. marinus* ammocoetes. The algae (diameter: $\bar{x} = 8.7 \pm 2.0$ μm S.D.) were grown in High Salt Medium (Sueoka, 1960), then washed via centrifugation and resuspension in filtered tap water. Control experiments indicated algal numbers did not measurably increase during experimental periods, presumedly because of low light levels. Percent efficiency of particle retention was assessed by two techniques. In both, algae were added to the anterior chamber of the tube device. In the first technique, the algal concentration was held constant for an interval during which a deflated balloon, fitted around the posterior end of the tube, collected the exhalent water. The balloon was never allowed to fill to the level where it exerted back pressure on the larva. Samples were removed from the balloon and the anterior compartment, diluted 4:1 with 0.1% NaCl, and their particles were counted six times with a Model FN Coulter Counter. Retention efficiency was calculated as:

$$RE = 1 - \frac{C_b}{C_a}$$

where $C_b$ and $C_a$ are the numbers of algae counted per ml from the balloon and anterior compartment samples, respectively.

In the second technique, no balloon was used. Here, the algal concentration in the anterior compartment decreased as the volume pumped from it by the ammocoete was replaced experimentally with clean water. Retention efficiency was calculated by the equation:

$$RE = \frac{C_{fp}V_{fp} - C_{ip}V_{ip}}{C_{ia}V_{ia} - C_{fa}V_{fa}}$$

where $C_{ia}$ and $C_{fa}$ are initial and final particle concentrations respectively in the anterior compartment, $C_{ip}$ and $C_{fp}$ are initial and final particle concentrations respectively in the posterior compartment, $V_{ia}$ and $V_{fa}$ are initial and final volumes of suspension respectively in the anterior compartment, and $V_{ip}$ and $V_{fp}$ are initial and final volumes in the posterior compartment. Again, particle concentrations were measured with the Coulter Counter.

Retention efficiency was calculated a total of 22 times, based on 7 individuals, for algal concentrations ranging from 1 to 75 mg (dry) per liter.

## RESULTS

Ammocoetes in the tubes usually remained still, wiggling ("crawling", Rovainen and Schieber, 1975) being infrequent. All the individuals exposed to *Chlorella* produced green feces about six hours after the presentation of food. Ammocoetes dug from the sand following exposure to yeast contained white cords within their guts, visible through the ventral skin. These findings suggest the larvae fed normally under experimental conditions. Burrows and tubes were never lined by mucus (*cf.* Sterba, 1953).

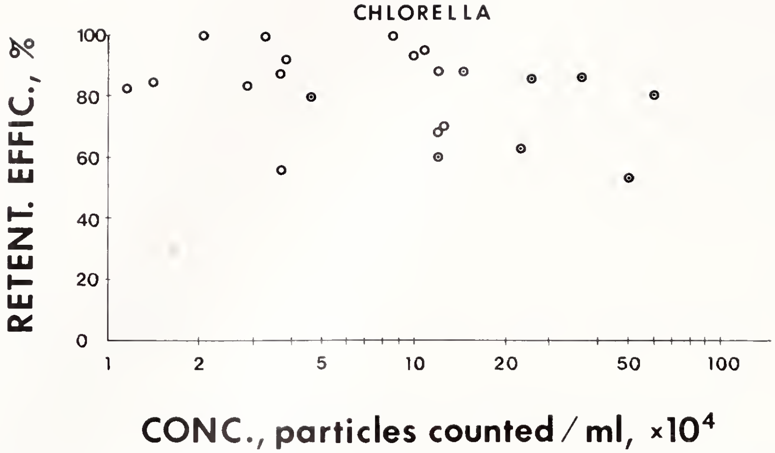

FIGURE 3. Percentages of *Chlorella pyrenoidosa* cells retained by larval *P. marinus* over a range of cell concentrations. Data pooled from seven ammocoetes. A count of $2.25 \times 10^5$ particles/ml corresponded to 27 mg dry mass per liter. The open dots represent values measured by collecting pharyngeal efflux in a balloon, while the closed dots were obtained by the dilution method described in the text. The least squares equation (linear) for all the points is:

$$RE = 86 - 2.94 \times 10^{-6}\, C, \qquad r = -0.33, \qquad P > 0.10.$$

Retention efficiency data are depicted in Figure 3. For concentrations of *Chlorella* between 1 and 75 mg/l, the fraction of particles removed was high, averaging 86 ± 13% S.D. and 75 ± 13% S.D. respectively, as measured by the balloon and dilution techniques. These means did not differ statistically from one another at the 95% confidence level ($t = 1.96$, $P > 0.05$), so the overall retention efficiency was calculated as 82 ± 14% S.D. There was no evidence that retention efficiency varied with algal concentration in the range studied.

Flow rates recorded for ammocoetes in the tubes are shown in Figure 4. One hundred and one hourly recordings, compiled from twelve individuals, ranged from 8 to 64 ml/g/h. The overall average hourly flow rate was 28 ml/g/h, with a standard deviation of 13. Individual average flow rates during the monitoring periods ranged from 10 to 52 ml/g/h in the twelve animals. Did flow rate tend to change with the amount of time spent in the tube? When both increases and decreases are considered, the mean hourly change did not differ significantly from zero (+3.2% ± 28% S.D., $P > 0.3$). Absolute hourly changes averaged 22%. Thus, although flow rates varied considerably over time, no consistent pattern of variation was evident.

Clearance rates recorded for burrowed ammocoetes are depicted in Figure 5. The quite similar results from the two species were combined. Mean clearance rates ranged from 3 to 13 ml/g/h, depending on the concentration of yeast in the overlying water, with an overall average of about 7 ml/g/h.

The placement of a 4-cm sand plug in the path of pharyngeal efflux of three ammocoetes in tubes led to decreases in flow over previous rates. Declines averaged about 50% (30 to 11, 23 to 15, and 19 to 11 ml/g/h).

## DISCUSSION

It could be suggested that confining ammocoetes within tubes affected flow rate, either physically—the glass walls interfering with movement of water out of the

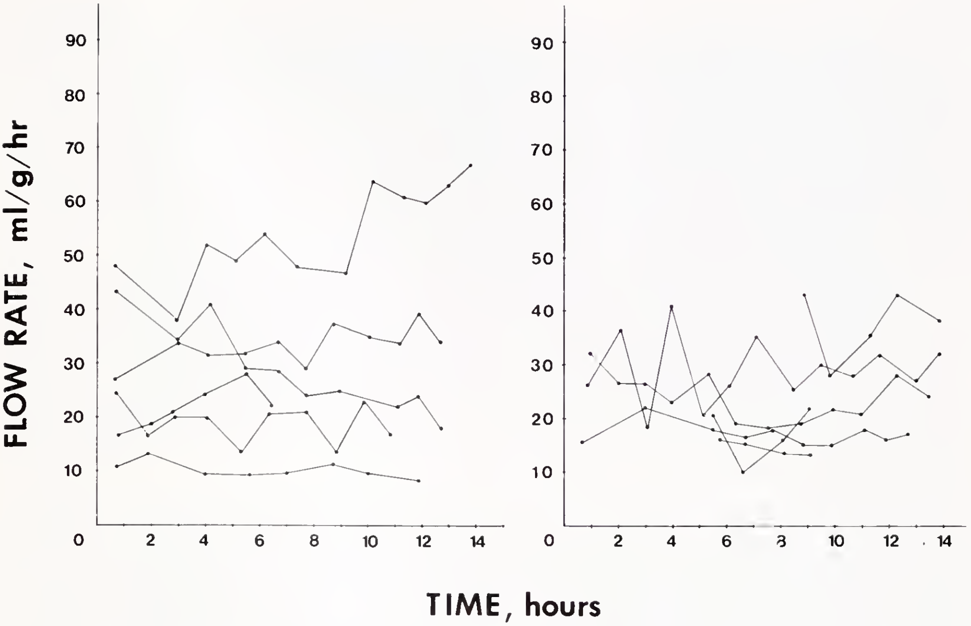

FIGURE 4. Variation in flow rate over time in twelve nonfeeding *P. marinus* larvae ($\bar{x}$ = 1.6 g), each within the device of Figure 2, at 12°C. Two graphs are used to avoid crowding. No consistent pattern of change is evident.

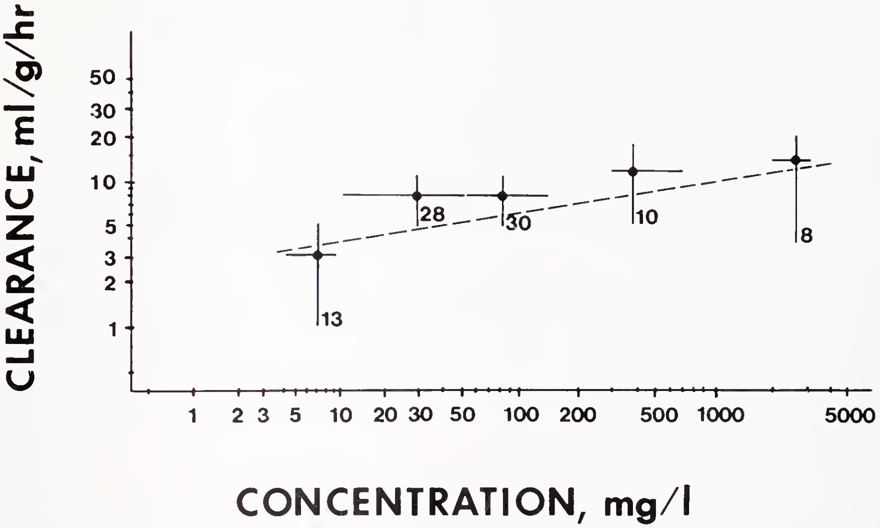

FIGURE 5. Response to yeast cells (*Saccharomyces cerevisiase*) of ammocoetes ($\bar{x}$ = 2.0 g) burrowed in sand, particle clearance rate vs. food concentration in the overlying water. Data from two species *P. marinus* and *L. tridentatus* were very similar (analysis of covariance, $P > 0.25$ for both slopes and intercepts of the log-transformed lines), and are combined. The least squares equations, calculated from the log-transformed data, are: for *Petromyzon*, $F = 2.9 \cdot C^{0.17}$ (46 points, $r = 0.29$, $P = 0.05$); for *Lampetra*, $F = 2.3 \cdot C^{0.25}$ (43 points, $r = 0.37$, $P < 0.05$); and for the combined data, as graphed, $F = 2.3 \cdot C^{0.21}$ ($r = 0.33$, $P < 0.01$). The dots represent mean values for points in the concentration ranges indicated by the horizontal bars. Vertical bars delineate 95% confidence intervals for clearance rates, and the numbers of points used in calculating these intervals are indicated below these bars. At 12°C.

gill pores—or behaviorally, through stressing the animals. Speaking against physical interference, it is noted that flow from the gill pores is normally posterior, not lateral (unpublished observations, on free animals presented concentrated carmine particle suspensions.). Speaking against stress, it is noted that larvae in tubes efficiently ingested food (Fig. 3), and seldom exhibited crawling behavior. The lack of any direction of variation in flow rate with time (Fig. 4) is also consistent with the view that the ammocoetes were not stressed. Indeed, placement in a tube seems to calm this burrowing organism (thigmokinesis: Hardisty, 1979, p. 56; the calming effect was also noted by Rovainen and Schieber, 1975).

Rovainen and Schieber (1975) validly point out that such tubes may interfere with cutaneous respiration, leading to a compensatory elevation of flow through the pharynx. The degree to which ammocoetes rely on cutaneous respiration is unknown (Lewis, 1980), although the thickness of the dermis suggests that the gills are much more important respiratory structures than the skin (Czopek and Sawa, 1971). Furthermore, opportunity for cutaneous respiration should be curtailed when ammocoetes occupy poorly permeable substrate. An overestimate of flow rates would not affect the conclusions of this paper.

The test of retention efficiency employed here measured the fraction of particles removed from suspension, not the fraction that actually entered the gut. It is conceivable that some error was introduced through the ammocoetes rejecting particles after filtration, or by some cells settling within the tubes and never reaching the balloon (although nothing was seen that indicated these things occurred). In future studies, the methodology will be expanded to include a quantification of gut contents.

Malmqvist and Brönmark (1982) determined clearance rates of *Lampetra planeri* ammocoetes in sand. Using the same technique, I obtained average clearance rates (7 ml/g/h) that are comparable to theirs (11 ml/g/h), considering that their animals were smaller (0.6, *cf.* 1.6 g) and the temperature, higher (15°C). However, those authors apparently considered their clearance rates to reflect flow rates, which may not be correct. In the technique employed, a mass of clean water, within the pore space of the sediment, is interposed between the ammocoetes and the overlying suspension. To the extent that the ammocoetes use this clean water, particles will not be cleared, and clearance rates will underestimate flow rates. That the discrepancy is significant is indicated by the observation that most ammocoetes in the test aquaria had closed burrows, cut off from the suspension overhead. As the flow rates of the burrowed ammocoetes in this study cannot be measured by clearance rates, they must be estimated by simulation. When pumping against a sand plug, whose length approximated the depth at which burrowed ammocoetes reside, larvae in tubes moved water at about half their unimpeded rate; thus, flow rates for the burrowed animals in this study are estimated as half those of unburrowed individuals, or about 15 ml/g/h.

In this study, ammocoetes filtered most ($\bar{x} = 82\%$) *Chlorella* particles from suspension over a range of concentrations, 1–75 mg/l, that should include those they experience in nature. Many suspension feeders begin to perform inefficiently when concentrations exceed 1–10 mg/l, rejecting particles (Jørgensen, 1975, pp. 64–65; Epifanio and Ewart, 1977). The evidence for efficient retention by ammocoetes at concentrations as high as 75 mg/l supports the hypothesis, proposed in the Introduction, that lamprey larvae are adapted to filter concentrated suspensions. The extensive system of feeding mucus may allow this.

Average flow rates, as measured for the animals in tubes, varied among individuals by a factor of five (10–52 ml/g/h). This large variation is noteworthy,

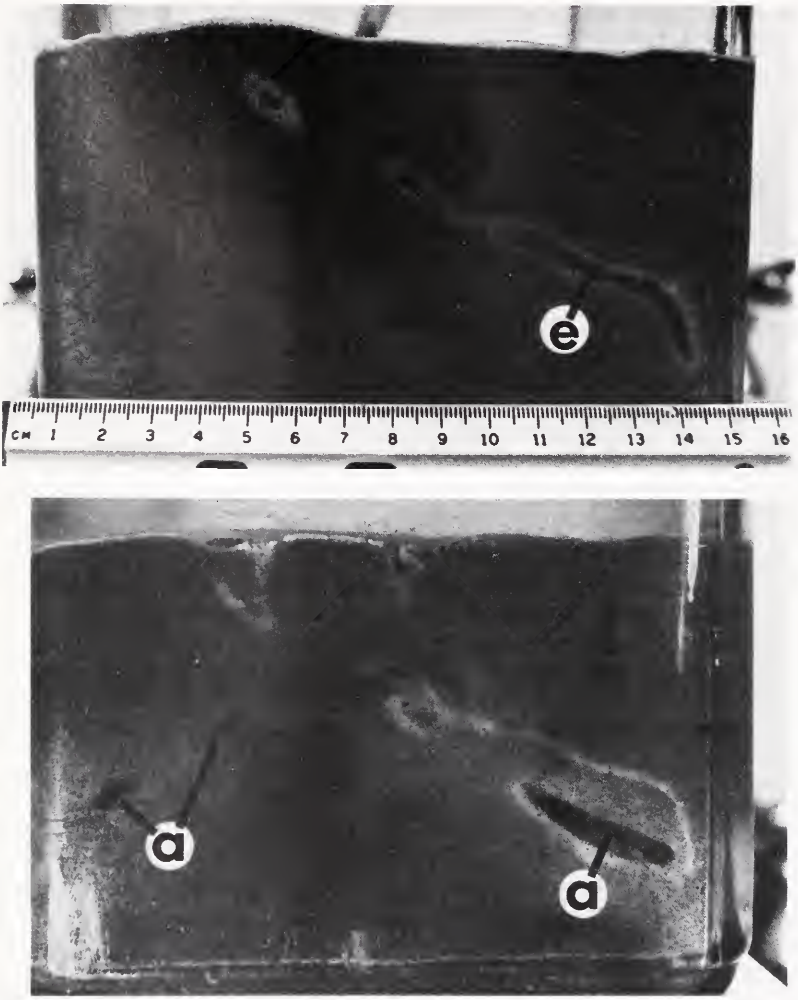

FIGURE 6. Photographs demonstrating that ammocoetes propel exhalent ventilatory water into the surrounding sediment. Above is shown an empty burrow (e) against the wall of an aquarium; below are two burrows containing ammocoetes (a). Most of the sand is dark, containing reduced organic matter. Above, a thin rim of light, oxidized sand outlines the empty burrow. This demonstrates the low permeability of the sand, aerated water having only diffused a few mm into it. Below, both ammocoetes (a) are surrounded by light halos, 1–2 cm thick, produced by their pharyngeal efflux. Such halos also surrounded ammocoetes buried in a very fine grained diatomaceous earth, although the light zones were thinner there. Oxidized zones also have been noted around infaunal deposit feeders (Aller, 1978, Fig. 1).

considering animal weights, temperature, and treatments were all standardized. Rovainen and Schieber (1975, their Table 1) also recorded large individual variation in flow rates; these ranged from 20 to 60 ml/g/h for undisturbed ammocoetes at 20° weighing 0.85–2.7 g.

In the present study, flow rates were calculated to average 28 and about 15 ml/g/h for unburrowed and burrowed ammocoetes ($\bar{x}$ = 1.6 g, 12°). Preliminary results from similar animals in tubes indicate that the presence of food (yeast) can increase flow rate by up to 50%, to about 50 ml/g/h (Mallatt, 1980). Even so, ammocoete flow rates are probably the lowest ever recorded for a suspension feeding animal, even when adjusted to compensate for the ammocoete's comparatively large size (Table I, fourth column). The low flow rates of ammocoetes are more like those produced by animals that do not depend on suspension feeding, such as macrophagous fish (Randall, 1970), infaunal deposit feeders (*Echinocardium* and *Malacoceras*: Foster-Smith, 1978), and some facultative suspension feeders (*Arenicola* and *Bithynia*: Jørgensen, 1966).

Several factors could be responsible for the low rate at which ammocoetes pump water. Most obviously, this should relate to the resistance of the substrate inhabited, which would preclude the evolution of a rapid flow rate. The quantity of intrapharyngeal mucus might also limit ammocoete flow rate, as do the fine mesh filters of some holotrich ciliates (Fenchel, 1980a,b).

This analysis reveals two peculiarities of ammocoete feeding. Compared to other suspension feeders, ammocoetes 1) pump water extremely slowly, and 2) are able to filter very concentrated suspensions. These are interrelated. Slow flow *allows* concentrated suspensions to be utilized in that, by presenting little food-carrying water to the filtering surfaces per unit time, it diminishes the tendency of these to saturate. Low flow rate also *demands* high food concentration, for only concentrated suspensions could be expected to fill nutrient needs when little food-carrying water is available per unit time.

An hypothesis of the ammocoete feeding strategy emerges from this analysis. Whereas most suspension feeders meet their food requirement by moving dilute suspensions rapidly across their feeding structures, ammocoetes cannot grow on dilute suspensions. A slow rate of water flow through the pharynx, necessitated by the high resistance of the substrate inhabited and the design of the pharyngeal pump, confines ammocoetes to environments where food suspensions are concentrated. Since the burrowing habit that limits flow rate is necessary for protecting lampreys from predation during the larval stage (Morman *et al.*, 1980), the requirement for concentrated suspensions seems basic to the animal's biology.

The peculiarities of ammocoete feeding could be of general ecological interest. The ability of infaunal animals to modify the chemistry of the substrate they inhabit has recently received much attention (Aller, 1980; Gust and Harrison, 1981; Lawrence *et al.*, 1982). For ammocoetes, which drive the overlying water directly into the sediment (Fig. 6), habitat modification could be considerable.

## ACKNOWLEDGMENTS

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# POST-LARVAL GROWTH OF *DISSODACTYLUS PRIMITIVUS* BOUVIER, 1917 (BRACHYURA: PINNOTHERIDAE) UNDER LABORATORY CONDITIONS

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## ABSTRACT

*Dissodactylus primitivus* is a small pinnotherid crab parasitic on spatangoid urchins. Post-larval growth has been observed in the laboratory in the absence of hosts. Individual animals were grown over the whole size range of the species for a period of 691 days after hatching. During growth of *D. primitivus* males and females, there was no significant change in carapace width with length. Relative to carapace width, male abdominal width increased isometrically. Growth of the female abdomen was allometric and could not be explained by a simple relationship. Two phases leading to sexual maturity were recognized: one of low positive allometry, the other of strong allometric growth. During the latter phase pubertal molts occurred.

Growth over time showed decreasing increments and an increase in intermolt periods, with slight differences between sexes. The resulting growth rates closely fitted power curves. Compared to females, growth of males decreased after the first year. This could explain the presence of larger females in natural populations.

Several growth relationships analogous to weight were demonstrated and the results discussed in relation to other Crustacea.

## INTRODUCTION

Studies of age and growth contribute to an understanding of population dynamics and to the elucidation of developmental processes. Among Crustacea, such studies have most often centered on species of commercial interest (Maucheline, 1977), including prawn (Forster, 1970; Wickins, 1976), lobster (Thomas, 1965; Ennis, 1972) and crabs (Weber, 1967). Little comparable information is available on crustacean species, especially crabs, in the low size range. *Dissodactylus primitivus* is a small pinnotherid crab with a maximum carapace width of less than 1 cm, living as a parasite on the spatangoid urchins *Meoma ventricosa* and *Plagiobrissus grandis* (Telford, 1978b, 1982).

Despite many attempts since the landmark paper by Kurata (1962) to comprehensively describe age and growth in crustaceans, one major problem has persisted. It is the difficulty of obtaining morphometric data for known-age individuals. Several studies of allometry have relied entirely on collections of wild specimens of unknown age. For example, Finney and Abele (1981) have analyzed changes of shape with size in a xanthid crab, *Trapezia ferruginea* and Williams *et al.* (1980) have compared size and shape relationships in three species of *Uca.*

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Abbreviations: ABDW, abdominal width; AGE, days after hatch; CL, carapace length; CW, carapace width; INCR, increment; INSTAR, instar number; INTMT, intermolt period; P-INCR, percent increment.

Similarly, Haley (1969) was able to correlate sexual maturity with external morphology in the ghost crab, *Ocypode quadrata*, from specimens of unknown age. Growth increments at successive molts have also been estimated from individuals of unknown age. Thus Sheader (1981) described increase in size in an amphipod, *Parathemisto gaudichaudi*, from wild specimens maintained in the laboratory. Data from tagged animals released and recaptured (*e.g.* Bennet, 1974) suffer from the same inherent uncertainty. Theoretically, the solution to the problem should be simple: culture the organisms from egg or known larval instars. With known-age individuals it is possible to analyze the two principal components of growth (Needham, 1964), namely, rate and change of form (differential growth). Although considerable success has been achieved in laboratory culture of shrimps and lobsters (Bardach *et al.*, 1972), few, if any, crabs have been successfully reared in continuous culture.

We have raised *Dissodactylus primitivus* from egg to adult size, providing a series of measurements for each of the thirty or more instars for individuals of precisely known age. Post-larval growth is examined here with two major objectives: the first to observe changes in form with reference to size, the second to investigate growth as it occurs over time. Larval development is described elsewhere (Pohle and Telford, 1983). This paper on post-larval growth thus completes a developmental study of *D. primitivus*.

## MATERIALS AND METHODS

*Collection of parental stock.* Ovigerous female crabs were collected with host spatangoid urchins, *Meoma ventricosa*, from sandy bottoms in 5–18 m of water, off the western coast of Barbados. Specimens were kept in 50-liter tanks until hatching occurred. Egg masses were periodically examined for maturity and possible protozoan or fungal infection. Newly produced egg masses are bright orange in color, becoming pale yellow towards the end of maturation. Hatching of zoeae for this study occurred between 9 and 11 p.m. on June 12, 1978.

*Rearing procedure.* At time of hatching a numbered series of 100 of the most vigorously swimming, positively phototactic larvae were pipetted into individual 120-ml glass jars ⅔ filled with sea water. Maintenance of larvae followed procedures outlined by Pohle and Telford (1983). Post-larvae were inspected daily, given live food, and transfered to new containers with about 90 to 120 ml fresh sea water (depending on size). Measurements were taken following every molt. Animals and containers were kept in a water table of running sea water to approximate temperatures to natural conditions.

*Culture medium and conditions.* Sea water fed into the laboratory was found to be inadequate even when filtered. Instead, fresh sea water was collected daily about 500 m offshore. In this way filtering or addition of antibiotics were found to be unnecessary.

For the entire rearing salinity ranged from 31.5 to 34‰ (mean 33‰). Temperature varied between 26.5 and 29.5°C (mean 28°C), about 0.5 to 1.0°C above the natural environment. A 14-h photoperiod was maintained.

*Food.* Larvae and early post-larvae were fed with newly hatched *Artemia* nauplii *ad libitum.* In addition, selected plankton of appropriate size, collected in daily trawls, was given as a food supplement. For later stages increasingly larger *Artemia* nauplii and planktonic organisms on which crabs readily fed were used.

*Measurements.* At each instar, specimens were measured live, twice, and the results averaged. After molting the exuvium was also measured. No significant difference was found between exuvial and live measurements.

Imminence of molting was apparent by a change in carapace opacity 1–2 days before exuviation. Exuviae were never eaten by any of the specimens.

The following measurements were made under the light microscope by calibrated ocular micrometer: maximum carapace width, dorsally and anteriorly; carapace length from vestigial rostrum to posterior margin, ignoring curvature; and width of abdomen at its widest point.

*Statistical analysis.* All regression lines were fitted by least squares analysis. Its major disadvantage is that error is assumed to occur in only one of the two variates and may result in a low estimate of slope (Gould, 1975). There are several other methods (Sokal and Rohlf, 1969) which consider error in both variates, but least squares was used here following arguments given by Brown and Davies (1972), Gould (1966), and Finney and Abele (1981): (1) it is easier to interpret and allows the use of standard tests of significance; (2) since most correlation coefficients ($r$) in this study are above 0.90, results with other methods should not be substantially different; and finally, (3) a comparison of methods by Brown and Davies (1972), using Doryline ants, has shown differences in results to be very small. This was at least partly attributed to the particular discontinuous growth pattern of the arthropod exoskeleton, where size differences between instars are much greater than for individuals of a given instar. The same argument applies here.

Zar (1968) raised objections to the widely accepted use of log transformations of power functions, suggesting instead the use of the curvilinear non-transformed model. This problem has not yet been satisfactorily resolved (Finney and Abele, 1981), but the conventional linear transformation used here has been recommended (Sacher, 1970).

In order to study changes in growth, a reference dimension which itself shows little or no change in growth rate is selected (Brown and Davies, 1972). In brachyuran crustaceans this is usually either carapace length (*e.g.* Finney and Abele, 1981) or carapace width (*e.g.* Barnes, 1968). A regression of these two parameters is often isometric (Warner, 1977), *i.e.* without significant change in ratios during growth. Width was chosen here because carapace length in *D. primitivus* is a less reliable measurement due to curvature along the longitudinal axis.

The power function $y = ax^b$ is known to biologists as the equation of simple allometry. It has found wide application in the analysis of growth (Gould, 1966). The theoretical basis claimed for this function by Teissier (1960), however, has not been universally accepted (Kidwell and Williams, 1956), and consequently it should not be considered a fundamental law of growth (Pasternack and Gianutsos, 1969). In this study linear ($y = bx + a$), semi-log ($\log y = bx + a$), and power functions (as log-log, $\log y = b \cdot \log x + \log a$) were applied to all data and that model which combined the simplest explanation of the data with the best possible fit was chosen.

For determination of allometric status, regressions were tested against either an isometric intercept standard of 0 for linear regression, or an isometric slope standard of 1 for power functions with a Students $t$-test (Sokal and Rohlf, 1969). Analysis of co-variance ($F$-test) was employed to compare slopes. Regression lines, statistics, and bivariate scattergrams were obtained by computer from programs in the Statistical Analysis System (SAS) package.

## RESULTS

### *Survival and mortality*

In the laboratory mortality was highest during the relatively short larval life, especially the megalopa, only 44 reaching the first crab instar (Fig. 1). The number

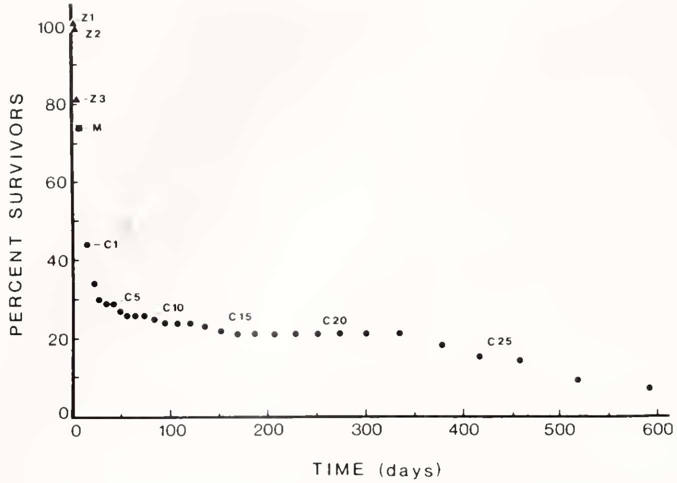

FIGURE 1. Survival of *Dissodactylus primitivus* in the laboratory. Points represent mean values for successive instars. Symbols: ▲, zoeal stages (Z1 to Z3); ■, megalopa (M); ●, post-larval instars (C1 to C28).

of survivors then gradually decreased, to 21 by instar 16 (mean day 169), with no further deaths occurring for about another ½ year (instar 23, mean day 335). Subsequently, numbers of survivors steadily decreased (a constant average mortality rate represented by points falling in a straight line) to 12 by post-larval instar 28, almost 600 days after hatching. On day 691 the rearing experiment was discontinued with 4 survivors remaining, 2 females (instars 28, 32) and 2 males (instars 28, 29).

### *Carapace width and length*

The relationship of mean carapace widths (CW) and lengths (CL) for post-larval instars 1–28 and of individual values for two specimens which reached instars 30 and 32 (Fig. 2) was given by the equation:

$$\mathrm{CL} = 0.828 \cdot \mathrm{CW} + 0.036 \qquad (r^2 = 0.994)$$

This calculated line, for the two sexes together, was based on 550 pairs of measurements. For separate sexes the intercepts were not significantly different from 0 ($t$-statistic = 1.02, $P = 0.31$ for males; $t = 1.81$, $P = 0.07$ for females). Thus the relationship was regarded as isometric.

The difference of slope between sexes was not significant ($F = 0.35$, $P = 0.55$). Male crabs (max CW = 8.1 mm), however, never reached sizes of the largest females (max CW = 9.5 mm). These large, laboratory-reared crabs are of equal or greater size than those found in the wild (CW < 10 mm).

### *Carapace width and abdominal width*

Based on 239 paired measurements of crabs from post-larval instars 5 to 28, the relationship of abdominal width (ABDW) and carapace width (CW) for male crabs was:

$$\mathrm{ABDW} = 0.389 \cdot \mathrm{CW} - 0.117 \qquad (r^2 = 0.997)$$

Fitting the data to a power function resulted in the equation:

$$\mathrm{ABDW} = 0.309 \cdot \mathrm{CW}^{1.010} \qquad (r^2 = 0.998)$$

Figure 3 shows a log-log transformation, where:

$$\log \mathrm{ABDW} = 1.010 \cdot \log \mathrm{CW} - 0.510$$

The slope of 1.01 indicated isometric growth ($t = 1.30$, $P = 0.21$).

During growth, abdominal width in females increased more in higher instars, fitting a power curve which, expressed as a log-log function, was given by the equation:

$$\log \mathrm{ABDW} = 1.481 \cdot \log \mathrm{CW} - 0.616 \qquad (r^2 = 0.984,\ \mathrm{N} = 226)$$

Regression slopes were significantly different for the sexes ($F = 1146.10$, $P = 0.0001$).

Analysis of growth of individuals and means for instars (Fig. 3) showed that the relationship was not of simple allometry. There was a change of slope approximately between instars 19 and 20 (see arrow Fig. 3). The data were better represented by two separate regressions, for early instars:

$$\log \mathrm{ABDW} = 1.295 \cdot \log \mathrm{CW} - 0.548 \qquad (r^2 = 0.995,\ \mathrm{N} = 157)$$

and for later instars:

$$\log \mathrm{ABDW} = 2.025 \cdot \log \mathrm{CW} - 1.048 \qquad (r^2 = 0.931,\ \mathrm{N} = 69)$$

Both slopes were significantly greater than 1 ($t = 55.14$, $P = 0.0001$ and $t = 35.42$, $P = 0.0001$, respectively) and hence growth was not isometric but positively allometric. The two slopes were significantly different ($F = 541.33$, $P = 0.0001$), indicating markedly different growth in the two size groups.

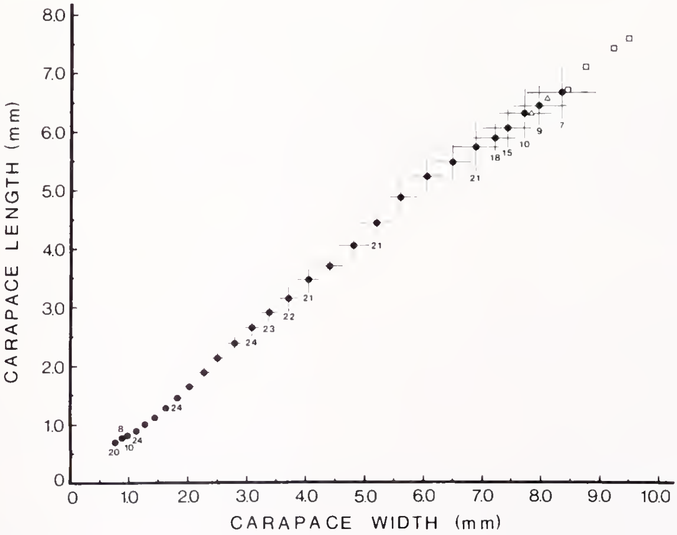

FIGURE 2. Relative growth of the carapace of *Dissodactylus primitivus*. Each circle represents a mean measurement for one instar up to instar 28. Number of observations per instar is shown; if not given, number equals that of adjacent instar. Vertical and horizontal lines are standard deviations. Additional points are given for the lone male (△) and the lone female (□) which surpassed instar 28.

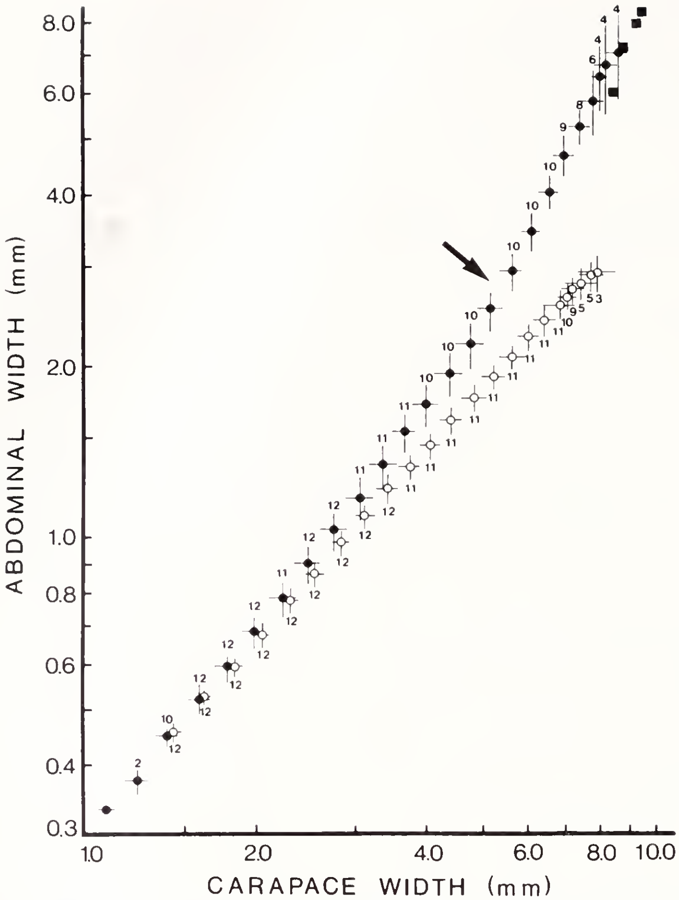

FIGURE 3. Relative growth of the abdomen of *Dissodactylus primitivus*. Open symbols represent males, closed symbols females. Each circular symbol is a mean measurement for one instar, with the number of observations given. Vertical and horizontal lines are standard deviations. Four additional square symbols represent instars 29 to 32 of one female individual in which molt of instar 28 to 29 represents a pubertal molt. Arrow between instars 19 and 20 indicates point of transition from early to late prepubertal growth.

### *Intermolt period*

Intermolt period (INTMT) and carapace width (CW) best fit a semi-log relationship (Fig. 4), as follows:

$$\text{Males: } \log \text{INTMT} = 0.127 \cdot \text{CW} + 0.722 \qquad (r^2 = 0.875, \text{N} = 260)$$

$$\text{Females: } \log \text{INTMT} = 0.110 \cdot \text{CW} + 0.767 \qquad (r^2 = 0.835, \text{N} = 250)$$

Slopes of these regressions were significantly different ($F = 15.66$, $P = 0.0001$).

A similar relationship was found between intermolt period and successive postlarval instar numbers:

$$\text{Males: } \log \text{INTMT} = 0.038 \cdot \text{INSTAR} + 0.671 \qquad (r^2 = 0.899, \text{N} = 293)$$

$$\text{Females: } \log \text{INTMT} = 0.034 \cdot \text{INSTAR} + 0.703 \qquad (r^2 = 0.859, \text{N} = 279)$$

Slope differences between sexes were significant ($F = 10.03$, $P = 0.0002$). Although these appear negligible, intermolt periods become significantly different at higher instars. For example, at instar 7 calculated intermolt periods were 8.6 days for males, 8.7 days for females and at instar 27 corresponding periods were 49.8 and 41.8 days. Regression equations for intermolt period and carapace width (above) yielded similar differences.

### *Growth increments*

Significant linear correlation was obtained between percent growth increments and carapace width or instar number using log-linear and linear-linear regressions, as obtained for other Crustacea by Maucheline (1977). Non-transformed regressions were chosen here, however, because of the significantly better fit for both sexes. Plotting percent increment (P-INCR) against carapace width (CW) (see Fig. 5) resulted in the equations:

$$\text{Males: P-INCR} = -1.464 \cdot \text{CW} + 14.894 \qquad (r^2 = 0.709,\ N = 256)$$

$$\text{Females: P-INCR} = -1.194 \cdot \text{CW} + 14.328 \qquad (r^2 = 0.734,\ N = 248)$$

An $F$ value of 13.16 indicated a difference of slope between sexes ($P = 0.0001$). Semi-log regression of the same data gave $r^2$ values of 0.605 and 0.698, respectively. A similar relationship was obtained between percent increment and successive instar

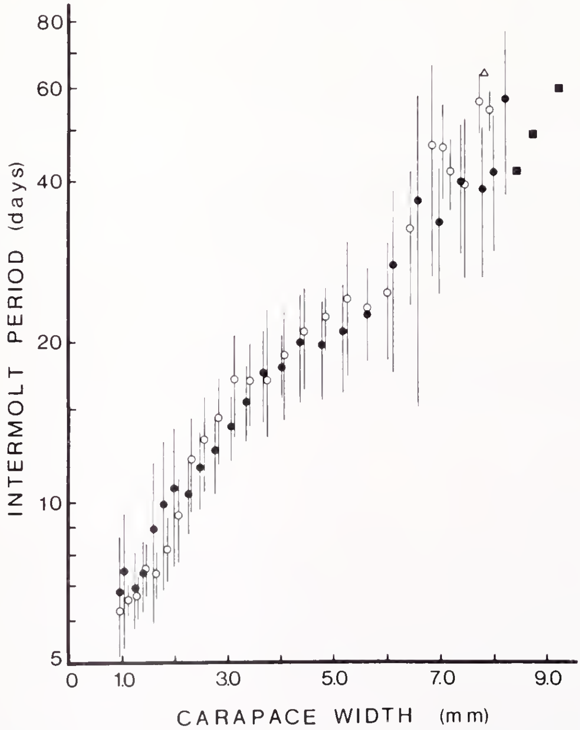

FIGURE 4. Intermolt periods and carapace widths of *Dissodactylus primitivus*. Symbols as in Figure 3. Vertical bars represent standard deviations. Additional intermolt periods are given for one male (△, instars 29–30) and one female (■, instars 29–32).

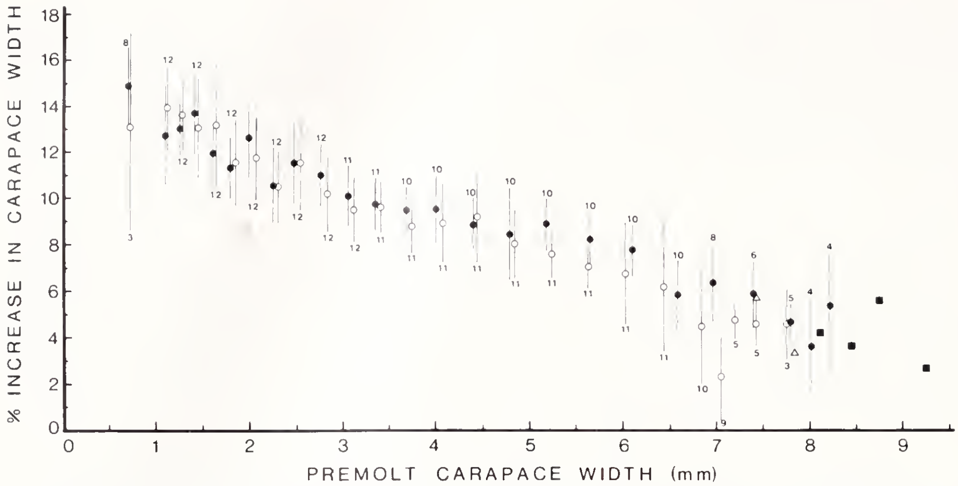

FIGURE 5. Carapace growth of *Dissodactylus primitivus* males and females as a percent increment based on premolt width. Symbols as in Figure 3.

numbers:

Males: $\text{P-INCR} = -0.449 \cdot \text{INSTAR} + 15.758 \qquad (r^2 = 0.707, \text{N} = 256)$

Females: $\text{P-INCR} = -0.382 \cdot \text{INSTAR} + 15.214 \qquad (r^2 = 0.729, \text{N} = 248)$

Semi-log regression resulted in lower $r^2$ values (0.556 and 0.664, respectively).

### *Growth rate*

The relationship between carapace width (CW) and age (days after hatch) is shown in Figure 6. For both sexes the data best fit a power function.

Males: $\log \text{CW} = 0.739 \cdot \log \text{AGE} - 1.053 \qquad (r^2 = 0.970, \text{N} = 268)$

and

$$\log \text{AGE} = 1.311 \cdot \log \text{CW} + 1.445$$

Females: $\log \text{CW} = 0.791 \cdot \log \text{AGE} - 1.172 \qquad (r^2 = 0.952, \text{N} = 260)$

and

$$\log \text{AGE} = 1.203 \cdot \log \text{CW} + 1.512$$

Slopes of male and female regressions were significantly different ($F = 14.46$, $P = 0.0002$). This seems to be primarily explained by the decreased carapace growth of older males. This is in agreement with observations that *D. primitivus* adult males are smaller than females in the wild. Growth in carapace width of individual females which passed through pubertal molts showed no significant departure from the curve (square symbols, Fig. 6). Thus, in contrast to abdominal width, carapace width and intermolt period does not seem to change abruptly at the onset of sexual maturity.

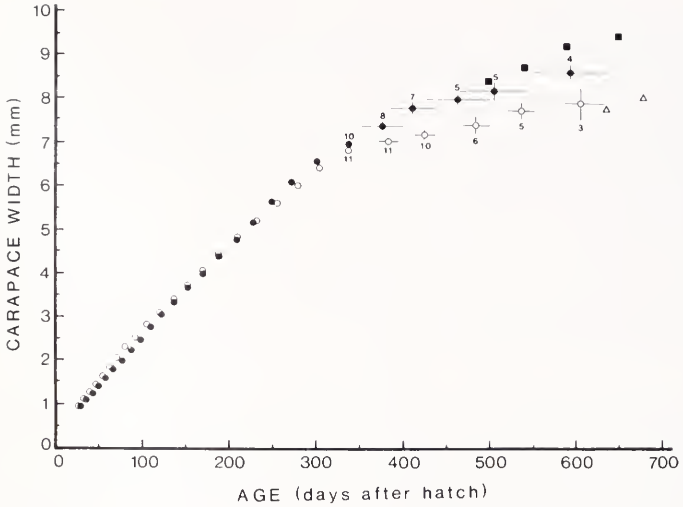

FIGURE 6. Growth rate of *Dissodactylus primitivus* males and females. Open and closed circles represent mean measurements of instars 3 to 28 for males and females, respectively. Additional points are given for one male (△, instars 29 and 30) and one female individual (■, instars 29 to 32). Vertical and horizontal lines represent standard errors for instars 24 to 28, with number of observations given. For remaining data points number of observations as in Figure 5.

### *Other relationships*

Growth of Crustacea can also be described by weight (Hewett, 1974). During this study weights were not recorded. Kurata (1962) and Maucheline (1977) pointed out that an analogous relationship to weight can be obtained by substituting the cube of body size measurements (carapace width or length) for weight. This holds true only in the absence of marked allometric growth. Studies of several Crustacea, including the spiny lobster *Jasus lalandei* (Fielder, 1964) and the king crab *Paralithodes camtschatica* (Weber, 1967) indicate that, for practical purposes, weight varies in direct proportion to the cube of carapace length ($y = ax^3$). On this basis several linear relationships have been reported.

Kurata (1962) showed a linear relationship between intermolt period and the cube of body length for several Crustacea. Regressing *D. primitivus* intermolt period (INTMT) against the cube of carapace width ($CW^3$) resulted in a linear relationship:

$$\text{Males: INTMT} = 0.092 \cdot CW^3 + 9.934 \qquad (r^2 = 0.775,\ N = 260)$$

$$\text{Females: INTMT} = 0.070 \cdot CW^3 + 10.436 \qquad (r^2 = 0.717,\ N = 250)$$

Although the difference between slopes was very small, it was statistically significant ($F = 28.57$, $P = 0.0001$).

In the common lobster, *Homarus vulgaris*, Hewett (1974) found that the log of growth increment, in weight, was linearly related to log of body weight. Maucheline (1977) obtained an analogous relationship for *H. americanus* by cubing log of increment (log $INCR^3$) and carapace length ($CL^3$) data. Using the same approach for *D. primitivus* with carapace width, a significant relationship can be obtained for the first 21 instars; for later instars there is significant deviation.

$$\text{Males: } \log \text{INCR}^3 = 0.584 \cdot \log \text{CW}^3 - 2.460 \qquad (r^2 = 0.667, \text{N} = 211)$$

$$\text{Females: } \log \text{INCR}^3 = 0.689 \cdot \log \text{CW}^3 - 2.548 \qquad (r^2 = 0.865, \text{N} = 207)$$

The difference in slope was significant ($F = 9.45$, $P = 0.0023$).

Hewett (1974) demonstrated that the log of body weight was linearly related to log of age. The analogous relation of the log of carapace width cubed (log $\text{CW}^3$) and log of age for *D. primitivus* was:

$$\text{Males: } \log \text{CW}^3 = 2.218 \cdot \log \text{AGE} - 3.158 \qquad (r^2 = 0.970, \text{N} = 268)$$

$$\text{Females: } \log \text{CW}^3 = 2.374 \cdot \log \text{AGE} - 3.517 \qquad (r^2 = 0.952, \text{N} = 260)$$

The slopes were significantly different ($F = 14.46$, $P = 0.0002$).

## DISCUSSION

In this study the highest death rate occurred during the short span of larval life, where it was due to imperfect molting. In the wild such high mortality has been observed but was attributed mostly to heavy predation (Warner, 1967). Data obtained in the laboratory suggest that mortality in the sea may also be at least partially caused by failure in molting. Post-larval deaths, although fewer, were also mostly attributed to molting difficulties similar to those described by Fielder (1964) for the spiny lobster, *Jasus lalandei*. Fielder noted that swelling of the new integument sometimes occurred before withdrawal from the old exoskeleton was accomplished. Thus, the time to complete a molt is limited. Similarly, either the appendages or the abdomen of *D. primitivus* could sometimes not be freed. Without exception all individuals in this study (larvae and post-larvae) molted at night and those which had not completed molting by daybreak died.

Changes in form of carapace occur in pinnotherid crabs symbiotic with pelecypods. *Pinnotheres ostreum* (Christensen and McDermott, 1958) and *Fabia subquadrata* (Pearce, 1966a), for example, have hard, flattened, or square invasive stages, respectively, followed by more convex or oval pre-swarming stages. These differences between stages are specialized adaptations to a life within molluscan hosts. In both *D. crinitichelis* (Telford, 1978a) and *D. primitivus*, carapace growth is isometric, length–width ratios not significantly changing over the size range. Such carapace growth is typical but not universal in crabs (Warner, 1977). Barnes (1968) found an increase in width over length during growth of some sentinel crabs (Ocypodidae). This change in shape is a functional adaptation to side-burrowing (Warner, 1977). Claims for various growth patterns are often made without adequate statistical testing (Brown and Davies, 1972). Re-examination of Barnes' data, for example, showed that only some of the species in fact had linear relationships with intercepts significantly different from zero (allometry). Finney and Abele (1981), studying growth in a xanthid crab symbiotic with corals, found that the carapace of males and non-ovigerous females increased in length over width, but ovigerous females showed isometric growth. Without suitable statistical tests such differences would not have been apparent.

Sexual dimorphism in *D. primitivus* is most apparent in abdominal growth. Males and females can be distinguished by abdominal widths during juvenile stages long before sexual maturity is reached. The increased abdominal growth for females is necessary in reproduction, where the abdomen acts in conjunction with the sternum as a cover to an incubation chamber. The abdomen of *D. primitivus* males grows more or less isometrically, whereas females show positive allometry. In other crabs (MacKay, 1943; Haley, 1973; Hartnoll, 1974; Finney and Abele, 1981) female abdominal growth cannot be explained by simple allometry with a single straight line. Two phases can be recognized: the first of high positive allometry (pre-puberty), followed by one of low positive allometry (post-puberty). When analyzing a population *en bloc*, the change in allometry is presumptive evidence of a pubertal molt, a sudden large increase in abdominal width indicative of sexual maturity (Haley, 1969; Finney and Abele, 1981). Data of *D. primitivus* suggest two phases leading to sexual maturity (Fig. 3). The first is a juvenile phase of low positive allometric growth, followed by one of stronger abdominal growth. It is during the latter phase that the species is capable of maturing, for it is here that pubertal molts for two females were observed (Fig. 3). Sexual maturity is thus not reached at a constant size (nor at a fixed instar) but varies from individual to individual. Hence the observed population inflection cannot be explained as a simple one-step process: a change of growth also occurs before the pubertal molt. Prior to successful reproduction various secondary sexual characters appear, and the internal reproductive system must become functional (Finney and Abele, 1981). The phase of increased abdominal growth probably marks one or more of these physiological changes before the pubertal molt. Haley (1973) observed two similar growth phases leading to sexual maturity in *Ocypode ceratophthalmus*. In that crab the second phase has been specifically attributed to increased growth of the fourth abdominal segment. After their pubertal molts, growth of the two *D. primitivus* female individuals decreased. Post-pubertal growth appears to represent a separate phase of abdominal development but has not been fully analyzed due to insufficient data. Abdominal growth in *Pinnotheres pisum* (Needham, 1950) also did not follow simple allometry. Needham fitted the data onto progressively higher polynomial functions in order to arrive at a continuous and accurate description of growth.

Compared to estimates for other Crustacea such as *Cancer magister* (Butler, 1961), the number of observed molts for *D. primitivus* to reach adult size seems high. On the other hand, the shrimp *Crangon crangon* (Meixner, 1969), had 23–25 post-larval molts before reproduction in females, and 22–25 in males. This is similar to *D. primitivus*, where pubertal molts occurred after 26 and 29 instars, respectively.

Growth of Crustacea can be described in terms of duration of successive intermolt periods, which increases in most Crustacea as the organism ages (Maucheline, 1977). There are some notable exceptions, and possibly there are also differences between sexes. Studies by Reaka (1979) on coral-dwelling stomatopods and Miller *et al.* (1977) on marine copepods seem to indicate a more or less constant molting frequency (isochronal development). In decapod Crustacea a difference in intermolt periods of equal-sized males and females was found by Meixner (1969) in *Crangon*, where large females molted more frequently. Large females of *D. primitivus* similarly showed shorter intervals between molts than did equal-sized males.

Duration of intermolt period is affected by several environmental factors, but especially by temperature (Lasker, 1966). Kurata (1962) showed that temperature variation significantly alters terms of the regression equations. In this study of *D. primitivus*, laboratory temperature was stable and similar to the natural environment.

Changes in size have commonly been analyzed in two ways. The widely accepted regression of post- on pre-molt body size (Hiatt, 1948), has recently been criticized (Maucheline, 1976, 1977) on theoretical grounds and because it presupposes constancy of growth increments. Alternatively, growth can be analyzed by plotting absolute size increase or percent increase against body size (Farmer, 1973). The former usually results in a positive relationship. However, the latter results in a negative relationship, percent increments decreasing with size. In place of body size, successive instar numbers may also be used with similar results.

Decreasing percent increments and body size can be fitted to straight lines (Maucheline, 1977) for many Crustacea, including lobsters (Fielder, 1964; Thomas, 1965)) and crabs (Warner, 1967; Turoboyski, 1973). Such a relationship was found for *D. primitivus*, but the mean growth increments were relatively small. In early stages they ranged from about 14 percent to near zero growth in later instars. Results here are comparable with such other pinnotherids as *Pinnotheres ostreum* (10% between instar 1 to 2, Sandifer, 1972), and *Pinnixa faba* and *P. littoralis* (about 20%, Pearce, 1966b). Larger species, such as *Cancer magister* (Butler, 1961), have considerably higher percent increments (43%, instar 1 to 2).

Growth increments, however, do not always fall on a single straight line. For the amphipod *Parathemisto gaudichaudi* Sheader (1981) showed two distinct phases: a juvenile phase of rapidly decreasing growth, followed by a maturing phase with more gradually decreasing growth. Ostracods and calanoid copepods are also believed to be exceptions (Maucheline, 1977). Miller *et al.* (1977) showed near constant percent growth increments for marine copepods.

Data for growth rates have been obtained in a number of ways (Burkenroad, 1950) including growth of tagged individuals, change in size-frequency distribution, and laboratory maintenance. Laboratory culture was chosen in this study because development of individuals could be followed for prolonged periods in controlled environments. While techniques differ, many studies have come to the conclusion that growth decreases with time, irrespective of size or species [Farmer (1973), and Hewett (1974) for lobsters; Meixner (1969) for shrimps; and Warner (1967), Weber (1967), and Bennet (1974) for crabs]. The data obtained from culture of *D. primitivus* support the above observation and fit a power function. This relationship has been indirectly estimated for other Crustacea (Warner, 1967) but never demonstrated by continuous long-term culture of individuals. Growth rates of *D. primitivus* are different for males and females (Fig. 6). During the first year these differences are slight, thereafter growth increments for males decrease (see equations for Fig. 5) and intermolt periods increase (see equation for Fig. 4). Thus the smaller size of males can be explained by a slower growth rate rather than by a cessation of molting. In wild populations of *D. primitivus* the same difference between the maximum sizes of males and females was observed.

Differences in growth rates between sexes are known for other Crustacea, most of which have larger males than females. In this respect *D. primitivus*, as other pinnotherids, is an exception. Size differences between sexes in pinnotherids living inside molluscs or burrows of polychaetes, such as *Pinnotheres ostreum* (Christensen and McDermott, 1958) and *Pinnixa cylindrica* (McDermott, 1981), have been attributed to differences in life history. Only male crabs may leave for another host in order to locate additional mates and thus become more vulnerable to predation. This results in fewer males reaching a larger size. In *D. primitivus*, which is an external parasite, it is likely that both male and female crabs move from host to host, just as in *D. crinitichelis* (Telford, 1978b). Larger *Dissodactylus* females may be necessary for the production of enough eggs to ensure propagation of the species.

Bennet (1974) suggested the use of a linear relationship between percent molt increment and premolt weight of the crab *Cancer pagurus*. Applying the cubed carapace length transformation, Maucheline (1977) found this relationship to be unsatisfactory at extremities of size for *Homarus americanus*. The data here also fit only a small part of the size range, and the relationship was rejected. However, a significant linear relationship was obtained with these parameters using a log–log transformation. For *Homarus vulgaris* Hewett (1974) obtained a significant linear correlation between log of intermolt period and the cube root of body weight. As Maucheline (1977) showed, this is analogous to log of intermolt period and body size (CW or CL), a relationship which has been demonstrated here.

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# THE CILIARY JUNCTIONS OF SCALLOP GILLS: THE EFFECTS OF CYTOCHALASINS AND CONCANAVALIN A

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## ABSTRACT

The ciliated junctions between the gill filaments of scallop gills were studied. Junctional cilia are borne on both sides of spurs of tissue—cilifers—extending from the filaments. In an intact junction, each cilium is paired with another cilium from a cilifer on a neighboring filament. An electron dense band underlies the plasma membrane of each junctional cilium along the line of apposition with its mate.

Cytochalasins A, B, and E caused gill test square preparations to break up into their component filaments. All three cytochalasins disrupted the electron dense band, and cytochalasins A and E also disrupted the ciliary microtubules. These effects were reversible.

The paired adhesion of the junctional cilia was also reversibly inhibited by treatment with Concanavalin A (Con A; 100 $\mu$g/ml). Con A bound to the surface of the junctional cilia was labeled with hemocyanin. After treatment with Con A alone, the label was lightly and evenly distributed over the shafts of the cilia, but was more densely concentrated at their tips. In cytochalasin-Con A preparations, the surface labeling of the junctional cilia increased with the duration of cytochalasin exposure.

## INTRODUCTION

The gills of filibranchiate bivalve molluscs, such as scallops and mussels are composed of curtains of filaments held in alignment by apposed patches of adherent cilia. These ciliary junctions are familiar structures, having been frequently observed and described in numerous species (Kellogg, 1890; Rice, 1901; Ridewood, 1903; Drew, 1906; Dakin, 1908; Gutsell, 1931; Atkins, 1937, 1938a,b; Mattei and Mattei, 1972; and Jørgensen, 1976). However, most of these many reports were primarily general descriptions of filibranch anatomy. Moreover, until recently (Mattei and Mattei, 1972), the observations were made by light microscopy, so the fine structure of the ciliary junctions remained unknown. This remains the case, although the morphology of the tip of a junctional cilium of the scallop has now been described in great detail (Dentler, 1980).

Ciliary junctions are examples of a ubiquitous set of phenomena, all involving cell-cell adhesion. A variety of systems have been used to characterize such interactions, from mating in yeasts (Taylor, 1964) and protozoa (*e.g.*, *Chalmydomonas*, Wiese, 1969, 1974; Snell, 1976a,b; and *Blepharisma*, Honda and Miyake, 1976), to the sorting out, aggregation, and reaggregation of various embryonic tissues and

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Abbreviations: CCA, CCB, CCE, cytochalasin A, B, E, respectively; Con A, concanavalin A; J-cilia, junctional cilia.

sponge cells (Humphreys, 1963; Steinberg, 1963; Moscona, 1965). We thought that the ciliary junctions of filibranch gills might be another useful system for investigating cell-cell adhesion, and that an examination of their pharmacology would provide an efficient first test of this possibility.

Two well-studied classes of drugs, the lectins and the cytochalasins, interfere with cell-cell adhesion. For example, cytochalasins A, B, and E all inhibit sponge cell reaggregation (Reed, *et al.*, 1976; Greenberg, *et al.*, 1977), and they also block adhesion of Ehrlich ascites cells to glass and plastic (Weiss, 1972). Cytochalasin B inhibits cell sorting and the spreading of embryonic chick cells (Steinberg and Wiseman, 1972), and it also blocks adhesion, aggregation, and spreading of platelets (Kay and Fudenberg, 1973).

In contrast to the cytochalasins, the plant lectin, Concanavalin A (Con A), interferes with cellular interactions by agglutinating the participant cells (Kapeller and Doljanski, 1972; Sharon and Lis, 1972). Some systems affected by Con A are reminiscent of bivalve junctional cilia. For example, Con A added to a culture of *Chlamydomonas* produces clusters of cells adhering at the tips of their flagella (see Fig. 1, Wiese, 1974; and McLean and Brown, 1974). In contrast, Con A and another lectin, phytohemeagglutinin (PHA), inhibit homotypic pair formation in *Blepharisma intermedium* by agglutinating the cilia (Ricci *et al.*, 1976). Finally, Con A tufted and clumped the cirri on the ciliate, *Stylonychia mytilus*, but reacted only weakly with two other ciliates., *Euplotes aediculatys* and *Tetrahymena pyraformis* (Frisch *et al.*, 1976).

In this report, we describe the ultrastructure of the ciliary junctions of scallop gills. Then we examine the effects on the ciliary junctions of the cytochalasins and Con A, alone and in combination. Both scanning and transmission electron microscopical observations were made.

Preliminary accounts of this work were reported to the Marine Biological Laboratory, Woods Hole (Greenberg, 1969), and the American Society of Zoologists (Reed and Greenberg, 1976).

## Materials and Methods

The experimental animals, *Argopecten* (= *Aequipecten*) *irridians*, were obtained from the Northeast Marine Specimens Company, Inc., Bourne, Massachusetts. They arrived in Tallahassee, Florida in good condition, and were kept in aquaria, in filtered, vigorously aerated natural seawater from the Gulf of Mexico (31 ppt), at 15°C. Under these conditions, the scallops survived for at least 6 days; but they were used for experimentation within 72 hours of their arrival.

### *Preparation of test squares*

The gills were dissected from the animal and placed in seawater. Small sections (1 $cm^2$), containing about 100 junctions, were cut from the centers of the gills. These "test squares" remained intact and active for about 24 hours, propelled about continuously and randomly by their feeding cilia.

Test squares were the starting material for all of the microscopical and pharmacological observations reported here.

### *Scanning electron microscopy*

Test squares, cut from the center of a gill, were placed directly in Parducz fixative (1% $OsO_4$:$HgCl_2$ = 5:1) for 10 minutes. The tissue was then dehydrated in a graded series of acetone solutions. Following the last dehydration step, the material was dried in $CO_2$ by the critical point method. The dried material was attached to SEM stubs with nail polish and coated in a Denton Model 502 vacuum evaporator with approximately 100 Å of gold-palladium. The specimens were observed with a Cambridge S4-10 scanning electron microscope operated at 20 KV.

### *Transmission electron microscopy*

Test squares were prepared as usual, and then fixed for 30 minutes in a 1% solution of gluteraldehyde in 0.1 *M* phosphate buffer, at pH 7.5. The squares were washed in the phosphate buffer, and fixation was then continued in 1% $OsO_4$ in 0.1 *M* phosphate buffer at pH 7.5. The fixed samples were dehydrated in a graded series of alcohols, and then taken through two changes of propylene oxide. The material was embedded in Epon 812-DER 736. Silver to gold sections were cut with a diamond knife on a Sorvall Porter-Blum MT-2 ultramicrotome, and were poststained with uranyl acetate and lead citrate. The specimens were observed with a Philips 201 transmission electron microscope operated at 60 or 80 KV.

### *Bioassay of cytochalasin and Concanavalin A*

Test squares were cut from the gill and placed in 10 ml of sea water in Syracuse dishes, one section per dish. Stock solutions (2 mg/ml) of cytochalasins A, B, and E (Imperial Chemicals Industries, Ltd., Macclesfield, Cheshire, U. K.) in dimethylsulfoxide (DMSO) were prepared, and aliquots taken up in a microliter syringe were transferred to the sea water in the Syracuse dishes to achieve the appropriate test concentration (*i.e.*, 5, 6, 7, 8, 9, or 10 $\mu$g/ml). The time between the application of a dose of cytochalasin, and the complete dissociation of a test square into its component filaments, was taken as a measure of the effect of that dose.

Concanavalin A (Con A; Miles-Yeda Ltd., Miles Laboratories, Kankakee, IL) activity was assayed in an identical manner to that described for the cytochalasins, except that the drug was dissolved in glass-distilled water. The test concentrations of Con A were 25, 50, 75, and 100 $\mu$g/ml.

### *Electron microscopic assessment of drug actions*

Test squares of the gill were incubated in the series of cytochalasin test concentrations in seawater. After 5, 10, or 15 minutes of incubation, the tissues were prepared for scanning (SEM) or transmission electron microscopy (TEM), as described above.

Similarly, test squares were incubated for 10 minutes in the Con A test solutions, rinsed in filtered sea water, and then fixed for SEM.

In order to rule out any fixation artifacts that could be interpreted as drug-induced effects, control (untreated) tissue was fixed for electron microscopy with every group of experimental tissue.

### *Incubation in cytochalasins followed by incubation in Con A*

Stock solutions (2 mg/ml) of the cytochalasins were taken up in a microliter syringe and transferred to 10 ml of sea water; the final concentration was 10 $\mu$g/ml. After 10 minutes in the cytochalasin solution, the gills were rinsed in sea water and 100 $\mu$g/ml Con A was added immediately. Following this second incubation (10 min) in Con A, the gills were rinsed and fixed for SEM.

### *Hemocyanin labeling procedure*

The test squares were fixed in Parducz solution, rinsed, incubated in 100 μg/ml Con A for 10 minutes, then rinsed again. This was followed by an incubation in 1 mg/ml hemocyanin (keyhole limpet; lyophilized powder, ammonium sulfate free; Calbiochem, La Jolla, CA). The tissue was then dehydrated and prepared for SEM as usual.

Since the amount and distribution of Con A binding depends on whether the tissue is fixed before or after exposure to the lectin, other test squares were prepared as described above, except that the incubations with Con A and hemocyanin preceded fixation in Parducz solution.

Controls for the hemocyanin labeling procedure were carried out with the hapten inhibitor of Con A binding, alpha-D-methylmannoside (Brown and Revel, 1976). The gill test squares were incubated for 10 minutes in 0.1 *M* alpha-D-methylmannoside, either with, or after, the Con A incubation, but before hemocyanin labeling.

The effects of cytochalasin incubation time on the number and arrangement of Con A receptors on the J-cilia were assessed by the following procedure. A set of test squares were incubated in 10 μl/ml cytochalasin. Sample squares were removed from the medium at 1-minute intervals, from 0 to 30 min. As the tissues were removed from the cytochalasin solution, they were fixed, incubated for 10 minutes in 100 μg/ml Con A, rinsed, incubated for 10 minutes in 1 mg/ml hemocyanin, rinsed again, and prepared for SEM as usual.

Control experiments to test the effects of drug solvents were run, but no effects were observed. The various drug actions described below were consistently observed on the junctional cilia, whereas the appearance of the rest of the gill tissue was the same, whether or not it was treated. Therefore, osmotic damage to the membranes during fixation, even if it occurred, could not have contributed to the results.

## RESULTS

The gills of scallops are parallel linear arrays of W-shaped filaments, suspended from their centers by the gill axis. The gills are plicate, or pleated, with the plicae (the pleats) occurring about every eighth to fourteenth filament. A large filament, the principal filament, is located at the apex of each plica (Fig. 1).

There are two types of cilia on the gill—the *feeding cilia*, distributed along the length of the filament; and the *junctional cilia*, located only on tongue-like projections from the filaments (Fig. 2). The feeding cilia create the feeding and respiratory water current, and sort and distribute the small particles borne on this incurrent stream. The feeding cilia are about 10 μm long.

In scallop gills, the tongue-like projections bearing the junctional cilia (or J-cilia) occur at intervals of 0.1 mm along the length of the filaments. The projections on adjacent filaments are in register, and overlap like a set of spoons (Fig. 1). We call these tongues *cilifers* (*i.e.*, "cilia-bearers"). The cilifers assume a variety of shapes ranging from round to elongate. The regular filaments have one cilifer per 0.1 mm of length, and all of the cilifers point in the same direction. In contrast, the principals have two oppositely directed (anterior and posterior) cilifers occurring

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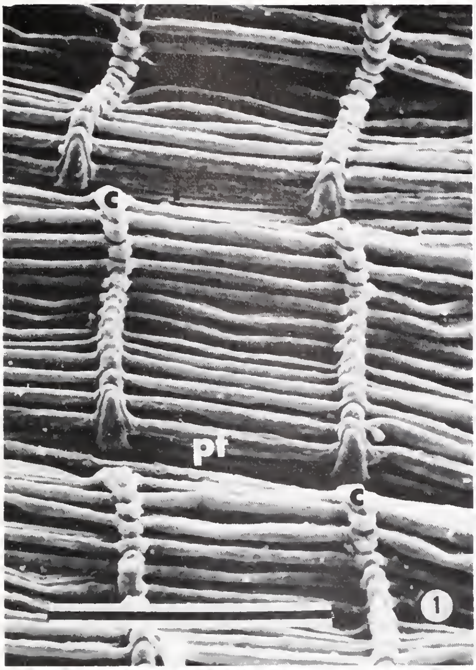
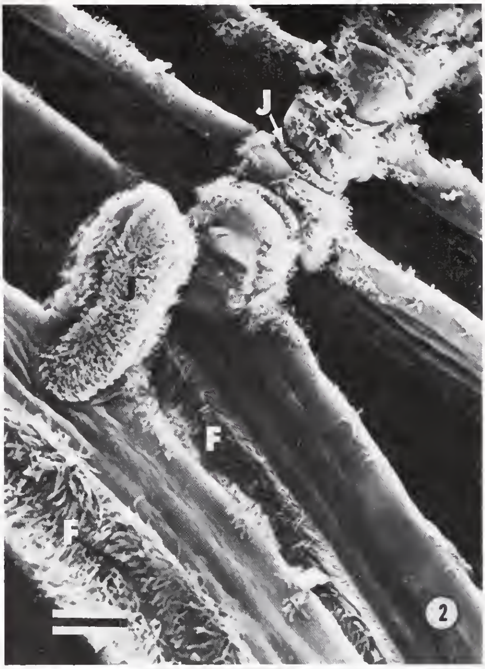
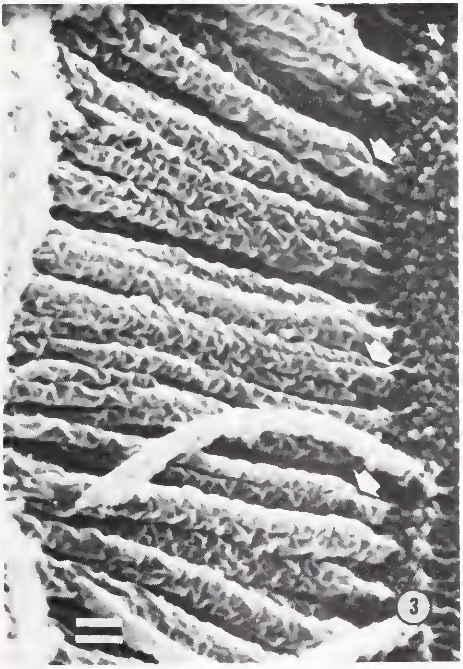
FIGURE 1. Scallop gill filaments with two rows of ciliary junctions. The cilifers (structures bearing the junctional cilia) are in rows perpendicular to the filaments. Note the plicated appearance of the gill. pf = principal filament; c = cilifer. Bar = 50 μm.

FIGURE 2. One of the rows of ciliary junctions oriented at right angles to the gill filaments. The junctional cilia (J) are visible between the intact junctions and on the single unpaired cilifer. The feeding cilia (F) are along the length of the filaments. Bar = 40 μm.







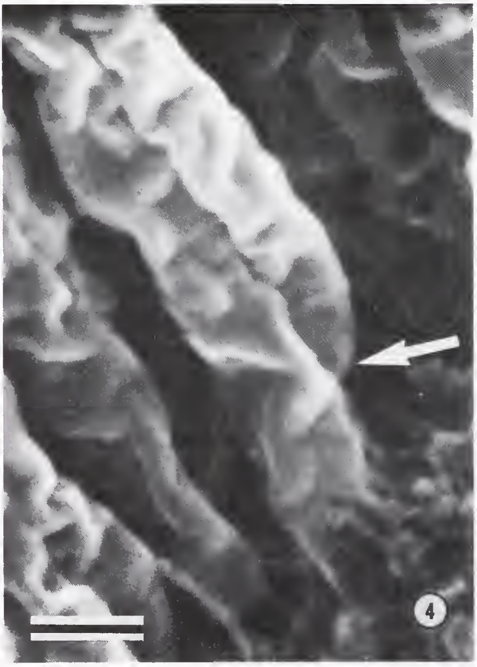


FIGURE 3. An intact ciliary junction. Each J-cilium is paired along its length with one from the opposite side of the junction. The tip of each cilium of a pair is hooked around the base of its mate (arrows). Bar = 1 μm.

FIGURE 4. Higher magnification of an area in Figure 3, showing the tip of one junctional cilium hooked around the base of its mate (arrow). Bar = 250 nm.

at 0.1 mm intervals (Fig. 1). Junctional cilia cover both sides of the cilifers on the regular filaments, but only one side of those located on the principal filaments. The J-cilia (6 $\mu$m long on the average) are shorter than the feeding cilia. There are about 800 cilia on each side of a cilifer, so the density is about $6.7 \times 10^3/\text{mm}^2$.

The surfaces of both the feeding and J-cilia are rough and wrinkled. Each J-cilium is closely apposed along its entire length to one other cilium from a cilifer on a neighboring filament (Fig. 3). Therefore, the cilia on the facing surfaces of the two cilifers making up one junction interdigitate. Moreover, the last 0.4 $\mu$m of the tip of each of these paired cilia is hooked around the base of its mate (Fig. 4). The pairing of the J-cilia is evident in TEM cross sections (Fig. 5). In such sections, the arms of the microtubules in each cilium of a pair are seen to be oriented in opposition to those of its mate, confirming that the two cilia arise from opposite cilifers. In addition, an electron dense band underlies the plasma membranes of adhering J-cilia along the line of their apposition (Fig. 5).

### *Dissociation of test squares by cytochalasin*

A sufficient dose of any of the three cytochalasins, A, B, and E, caused test square preparations of scallop gills to dissociate into their component filaments. The uncoupled filaments swam around the dish propelled by their feeding cilia; they collided, but never stuck, even when two cilifers made contact. If the cytochalasins were removed by replacement of the solution with normal medium, then cilifers making random contact would adhere, and mats of filaments would occur.

The dissociation of the test squares was dependent on three factors: the dose of cytochalasin used; the time that the cytochalasin was left in contact with the preparation; and the cytochalasin being tested. At any dose, cytochalasin E was more potent than cytochalasins A or B; the latter were equiactive (Table I).

### *Ultrastructural effects of the cytochalasins*

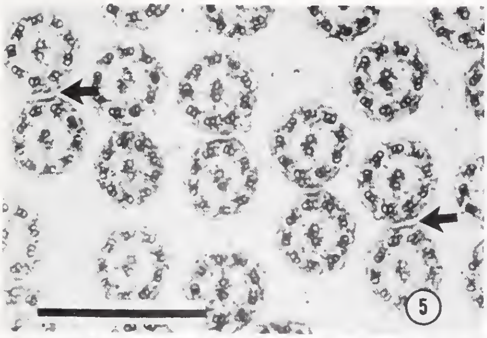
The J-cilia on the surface of mechanically isolated, but otherwise untreated, cilifers are uniformly unpatterned (Fig. 6, control). Cytochalasin treatment changed this picture. The first noticeable effect was the loss of randomness, and the formation of tufts of from 10–20 cilia (Fig. 7). Within each tuft, the cilia were joined only along their shafts; the tips were free and were often hooked over, or even curled into small knots. When the dose of cytochalasin was high, or the incubation time was long, a swelling or blebbing of the cilium appeared just below the tip. In some cases, and always at low doses of cytochalasin, the material was fixed before the test squares had completely dissociated into their constituent filaments; yet tufting at these intact junctions was already occurring (Fig. 8).

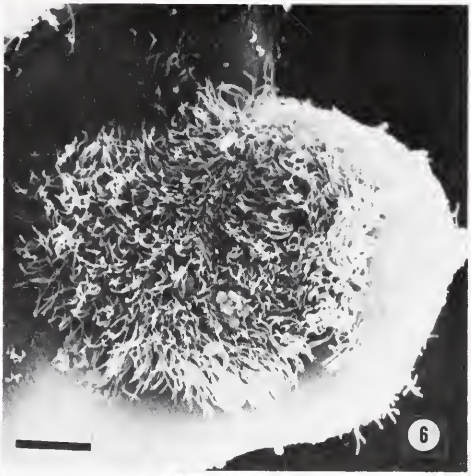
TABLE I

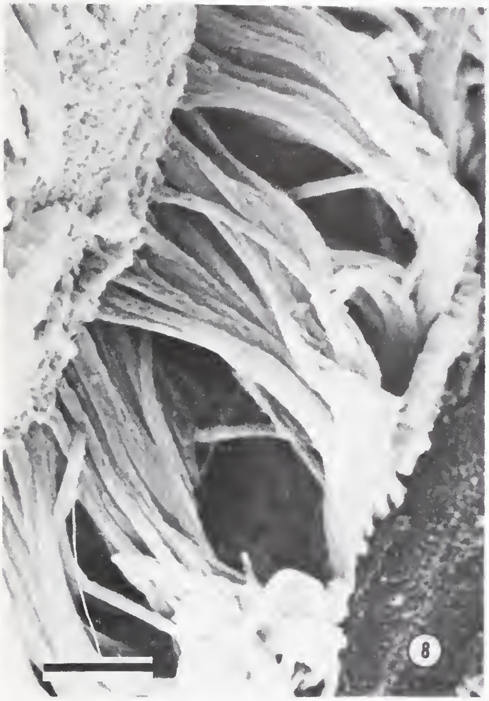
*The rate of scallop gill dissociation produced by the cytochalasins increases with dose.*

| | Dose ($\mu$g/ml) | | | | | |
|---|---|---|---|---|---|---|
| Cytochalasin | 5 | 6 | 7 | 8 | 9 | 10 |
| A | 31.5 | 26 | 24.5 | 21 | 18 | 14 |
| B | 31 | 31.5 | 24 | 20 | 13.5 | 8.5 |
| E | 24.5 | 18 | 15.5 | 14 | 11 | 6.5 |

Five sections of scallop gill (1 $\text{cm}^2$) were tested at each dose; the mean time (min) required for dissociation into individual filaments is tabulated.







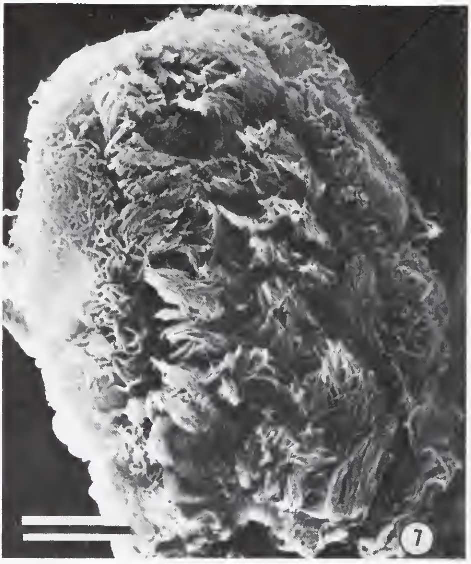


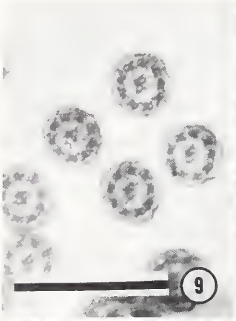
FIGURE 5. A transmission electron micrograph of a cross section through an intact junction, showing the pairing of the J-cilia and the electron-dense band underlying the ciliary membrane (arrow). Bar = 500 nm.

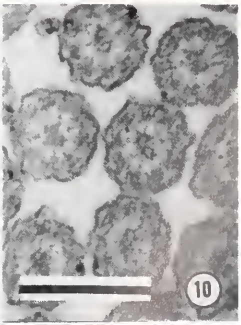
FIGURE 6. An untreated, control cilifer showing the unpatterned arrangement of the junctional cilia over the surface. Bar = 10 $\mu$m.

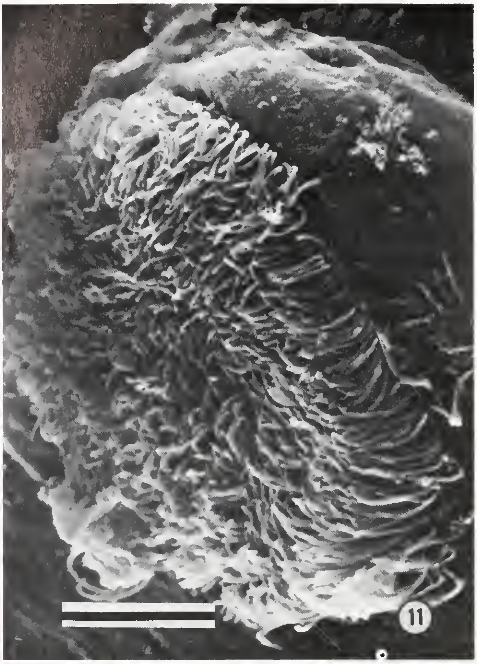
FIGURE 7. A cilifer after exposure to 10 $\mu$g/ml cytochalasin E for one hour. Note the tufting of the J-cilia. Compare with Figure 6. Bar = 15 $\mu$m.

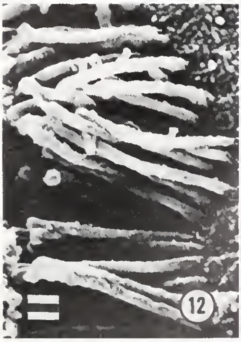
FIGURE 8. A ciliary junction following treatment with 10 $\mu$g/ml cytochalasin A. The two cilifers making up the junction are attached by tufted junctional cilia. Bar = 1 $\mu$m.

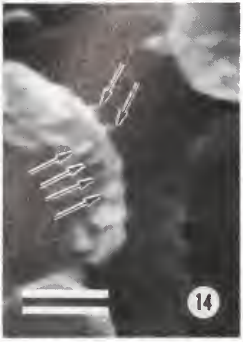
Transmission electron microscopy showed two additional effects of the cytochalasins on the J-cilia: the electron-dense band underlying the membrane of each J-cilium, on the side apposed to its mate, disappeared (Fig. 9); and the microtubules were poorly defined, and almost muddy in appearance following the administration of either cytochalasin A or E (Fig. 10). The disruption of the microtubules occurred only at relatively high doses of cytochalasin A or E (about 15 $\mu$g/ml),

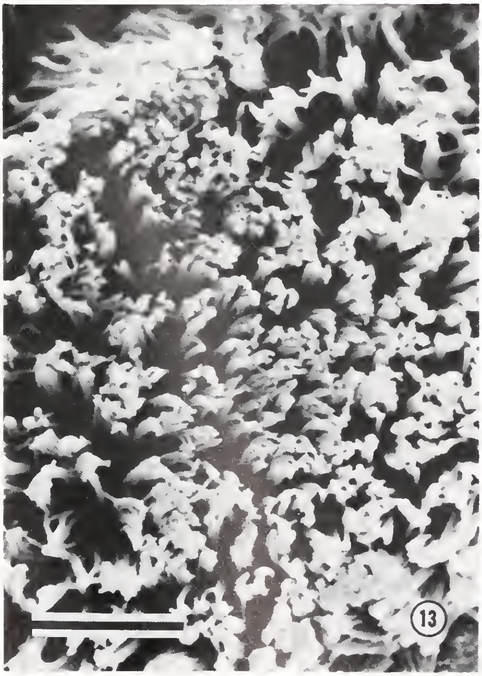












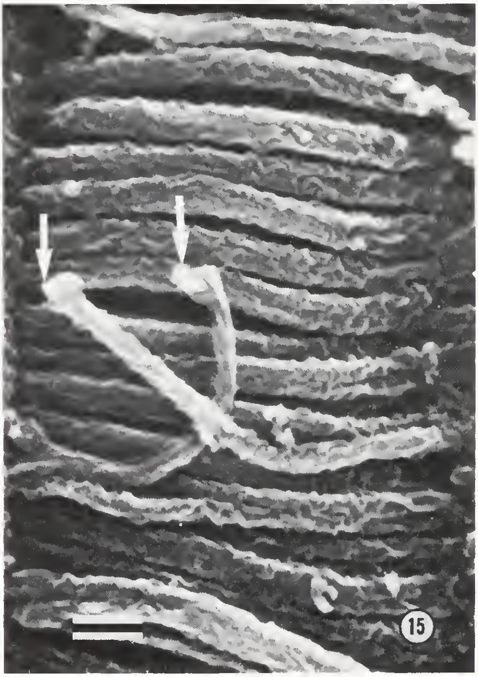


FIGURE 9. A transmission electron micrograph of J-cilia following treatment with 15 $\mu$g/ml cytochalasin B. The microtubules of the unpaired J-cilia are well defined, but the electron dense band is absent. Bar = 500 $\mu$m.

FIGURE 10. A transmission electron micrograph of J-cilia following treatment with 15 $\mu$g/ml cytochalasin E. The tubules and their arms appear muddy. Bar = 250 nm.

whereas lower doses (about 5 μg/ml) were sufficient to effect the disappearance of the electron-dense band. The microtubules were not affected by any dose of cytochalasin B.

In contrast to the J-cilia, the feeding cilia were apparently unaffected by the cytochalasins, even after large doses.

### *Effects of Concanavalin A*

Treatment with 100 μg/ml of Con A for 10 minutes usually caused test squares to separate into their component filaments. But in some tests, the dissociation was not complete, and some ciliary junctions remained coupled. The major effect of Con A on the junctional cilia was the production of tufts of from 10–20 cilia over the entire face of the cilifers (Fig. 11). But these tufts, in contrast with those produced by the cytochalasins, were clumped only at their tips; the shafts of the cilia were free from contact with their neighbors. Frequently the tips (1.5–2.0 μm) of the J-cilia were swollen 3–4-fold by treatment with Con A (Fig. 12). The same morphological changes were found following exposure to 25, 50, or 75 μg/ml Con A for 10 minutes.

### *Combined effects of Con A and the cytochalasins*

The 10-minute preincubation with 10 μg/ml cytochalasin caused the test squares to disperse into individual filaments. When these filaments were rinsed and treated for 10 minutes with Con A, the J-cilia again tufted into groups of from 10–20 cilia (Fig. 13). But instead of clumping only at their tips as with Con A alone, or clumping only along their shafts as with cytochalasin alone, the J-cilia were very closely apposed to one another along their entire lengths.

### *Distribution of Con A binding sites*

If gills were first fixed for SEM, and then exposed to Con A and hemocyanin, label was evenly distributed over the shafts of the unpaired J-cilia (Fig. 14). Those J-cilia that remained coupled to their mates on opposing cilifers were virtually unlabeled (Fig. 15). Similarly, only a small amount of hemocyanin was distributed over the feeding cilia and the rest of the gill tissue, probably labeling some mucopolysaccharide that had survived the fixation.

If the gills were treated with Con A for 10 minutes before fixation and labeling, the distribution of label was the same, but the density was lower. Again, there was little label on those J-cilia that were still paired (Fig. 16); but it was greater than on coupled preparations that had been fixed before being exposed to Con A and hemocyanin.

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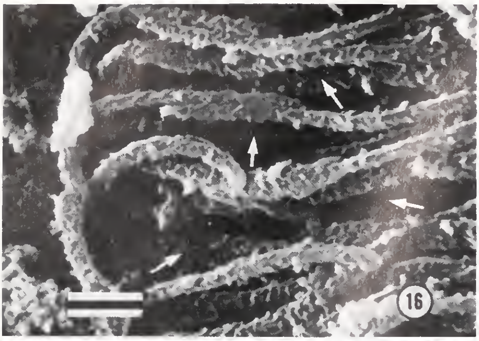
FIGURE 11. A cilifer after treatment with Con A showing the tufting of the J-cilia over the surface of the cilifer. Compare with Figures 6 and 7. Bar = 10 μm.

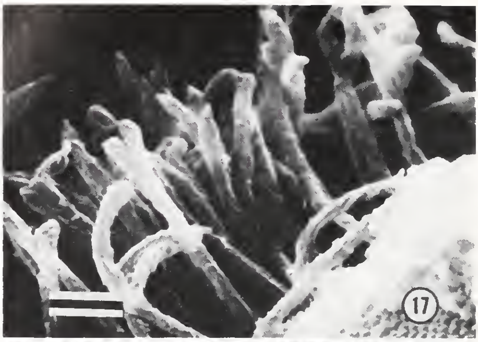
FIGURE 12. Tufted cilia on the outside edge of a cilifer after treatment with Con A. The cilia do not make contact at their shafts; the only area of union is at their tips. Bar = 1 μm.

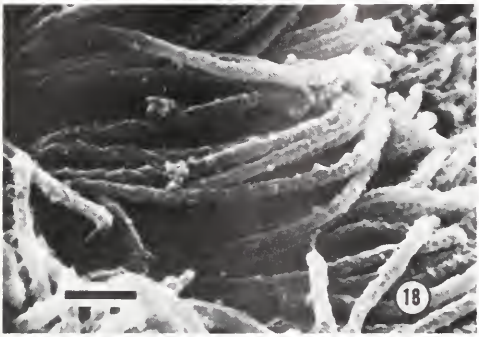
FIGURE 13. A cilifer after treatment with 10 μg/ml cytochalasin E for one hour followed by 100 μg/ml Con A for 10 minutes, showing the tufting of the cilia. Compare with Figures 6, 7 and 11. Bar = 10 μm

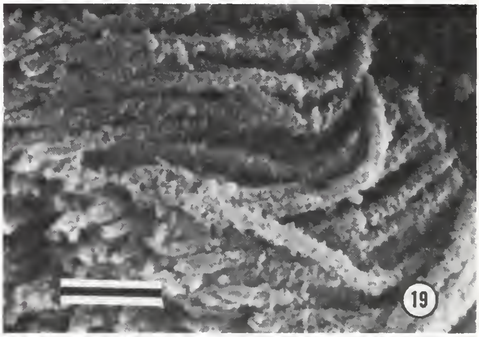
FIGURE 14. The distribution of hemocyanin label on the shafts of J-cilia (arrows). The tissue was fixed, then incubated with Con A and hemocyanin. Bar = 250 nm.

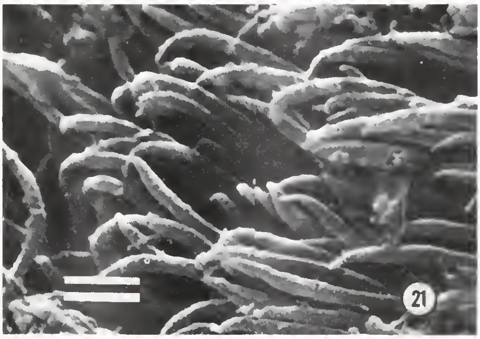
FIGURE 15. A junction after fixation and treatment with Con A and hemocyanin. One ciliary pair has separated, and hemocyanin label is present on the tips of the separated cilia (arrows). Bar = 1 μm.

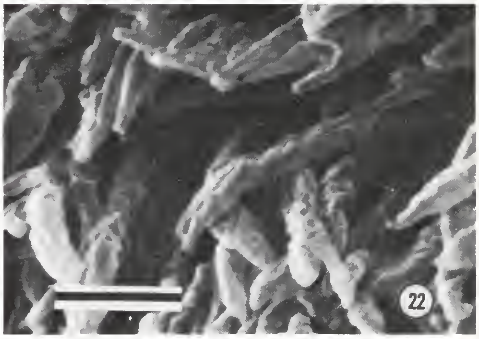












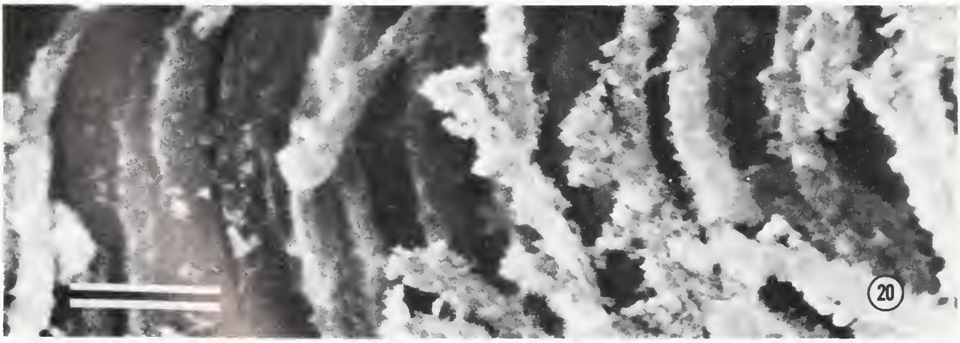


FIGURE 16. A partially intact junction fixed after a 10-minute exposure to cytochalasin. The partially coupled J-cilia have some label; underneath them are J-cilia which remain paired and unlabeled (arrows). Bar = 1 $\mu$m.

FIGURE 17. Junctional cilia fixed and simultaneously treated with cytochalasin, and then labeled. There is no label on the surface of the cilia. Bar = 1 $\mu$m.

In summary, J-cilia fixed before exposure to Con A and hemocyanin had less label than those fixed after labeling. In either case, there was very little labeling on paired J-cilia.

### *The effect of the cytochalasins on the distribution of Con A binding sites*

The density of labeled Con A binding sites on the J-cilia varied directly with the duration of exposure to the cytochalasins before fixation. When cytochalasin was added with the fixative (0 time), no label bound to the J-cilia (Fig. 17); moreover, there was no discernable increase in labeling at incubation times of up to 4 minutes. But if the tissue was preincubated with cytochalasin for 5 minutes, the amount of label increased noticeably from that observed at 0 time (Fig. 18), and the hemocyanin marker was widely dispersed over the entire ciliary surface. The label was denser after 10 minutes of exposure to cytochalasin; after 15 minutes, almost the entire ciliary surface was obscured by the heavy hemocyanin label (Fig. 19). Longer incubation times would not increase this response. No label was found anywhere on those J-cilia that remained paired (Fig. 20).

As a control for the cytochalasin-Con A experiments, gills were exposed to cytochalasin, followed by incubation with Con A and alpha-D-methylmannoside, a hapten inhibitor for Con A. Following this treatment, there was little, if any label present on the J-cilia, and they did not tuft or clump (Fig. 21). If the gills were incubated only with alpha-D-methylmannoside and Con A, the J-cilia tufted and were united at their tips (Fig. 22).

## Discussion

Three salient features emerge from our observations of the ultrastructure of *Argopecten* ciliary junctions. First, only the cilia of apposed cilifers are adherent; the cilia on isolated cilifers do not adhere. Second, the cilia adhere in pairs. Third, the membranes of adherent paired cilia (but not of detached J-cilia) are modified along the line of their apposition into thickened, electron-dense bands. These characteristics, particularly the narrow electron-dense band, have also been observed in the ciliary junctions of *Mytilus perna* (Mattei and Mattei, 1972), and they are probably common to ciliary junctions throughout the Bivalvia.

The characteristic pairing of adherent cilia, and their membrane modification, precludes the possibility that the adhesion could be due simply to frictional resistance between the tightly interdigitated sets of apposed cilia. Rather, these structural features suggest that the mechanisms of adhesion must include some specific molecular interaction between J-cilia. This conclusion is supported by two observations of Murakami (1962, 1963): that the J-cilia, whether dissociated or paired, are not stiff (*e.g.*, like hair brush bristles), but are flexible and motile; and that the connecting force of the junction is dependent on the ionic composition of the me-

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FIGURE 18. Part of a cilifer fixed 5 minutes after the addition of cytochalasin, then labeled. There is some label on the cilia. Bar = 1 μm.

FIGURE 19. J-cilia fixed after 15 minutes of exposure to cytochalasin, then labeled. There is labeling all over the ciliary surface. Compare with Figures 17 and 18. Bar = 1 μm.

FIGURE 20. A junction treated for 10 minutes with cytochalasin, then fixed and labeled with Con A - hemocyanin. There is little label on those J-cilia which remain paired, but the uncoupled J-cilia are heavily labeled. Bar = 1 μm.

FIGURE 21. J-cilia exposed to cytochalasin for 15 mintues, then incubated with Con A and alpha-methylmannoside and labeled with hemocyanin. There is little, if any label on the cilia. Bar = 1 μm.

FIGURE 22. J-cilia after incubation in alpha-methylmannoside and Con A (10 min). The cilia are tufted and adhere at their tips. Bar = 1 μm.

dium. The disruption of ciliary junctions by the cytochalasins and Con A also suggest a molecular, rather than a mechanical, adhesion.

The above argument notwithstanding, a few additional observations imply that the adhesion between J-cilia has a mechanical component as well as a chemical one. First, the mutual hooking of each ciliary tip around the adjacent complementary ciliary base could be providing a mechanical link. Second, the basis for such a linkage is suggested by a recent ultrastructural study of ciliary tips, including those of the J- and feeding cilia of *Argopecten* gills (Dentler, 1980). The J-cilia tips are unique in that their central microtubule caps are connected to the outer tubule doublets by the distal filaments. This arrangement results in a direct mechanical connection between the distal-most patch of ciliary membrane (attached to the central microtubule cap) and the outer doublet. Given this connection, together with the structural discontinuity in the cilium at the ends of the outer doublets, differential shortening of the doublets (Satir, 1968) might well cause the tip of the cilium to hook over. Third, low doses of cytochalasins sometimes produce tufting while the junction is still intact; and in such instances, the tips of the paired cilia remain wrapped around their mates, but away from the base, *i.e.*, towards the middle of the shaft. Thus, the proposed mechanical link seems to slip under stress and to be separable from the adhesion occurring along the paired shafts. Finally, Murakami (1962) showed that when previously separated cilifers of *Mytilus* were held together, they would adhere after "several minutes," but the force of adhesion would increase to a maximum by about 10 hours. This observation, which we have repeated qualitatively on scallop gills, is suggestive of at least two adhesive processes, one occurring rapidly, and one developing more slowly. We cannot, at present, identify the components, and other interpretations of the data are of course possible; yet we conclude that a mechanical contribution to adhesion is a reasonable possibility that should remain open.

The ciliary junctions of bivalve filibranch gills are reminiscent of heterotypic interactions between cilia of Protozoa. For example, the early stage of conjugation in the ciliate, *Blepharisma intermedium*, is characterized by a ciliary union between two cells, one from mating type I, and another from mating type II (Honda and Miyake, 1976). Again, conjugation in *Chlamyodomonas* is initiated by contact between the tips of flagella of sexually different gametes (Wiese, 1969, 1974).

Homotypic ciliary complexes are also seen in the Protozoa, particularly in the adoral zone of membranelles, the undulating membranes, and the cirri of ciliates. The cilia in these complex structures are closely apposed, like those of the bivalve ciliary junction, but they are not paired; and the membrane modifications include numerous small projections along the shafts (Roth, 1956; Randall and Jackson, 1958; Giese, 1973), and parallel rows of intramembrane particles at the tips (Montesano *et al.*, 1981). Such complexes are most similar to the latero-frontal cirri, feeding organelles of mussel and oyster gills (Owen, 1974; Owen and McCrae, 1976). Finally, the adhesion between the flagellum and the body wall of a trypanosome (the "undulating membrane") is mechanically dissociable and seems to involve membrane modifications (reviewed by Hoare, 1972). This system is thus reminiscent of J-cilia adhesion.

### *Effects of the cytochalasins*

Four ultrastructural changes appeared following the application of the cytochalasins. First, all three of the compounds tested—cytochalasins A, B, and E—eliminated the electron-dense bands in the apposed membranes of paired cilia. One of the classical mechanisms of action of the cytochalasins is the depolymerization of actin-like microfilaments (Wessels *et al.*, 1971). For example, such filaments found in BALB and 3T3 cells disappear after cytochalasin treatment (Gershenbaum *et al.*, 1974), and the microfilament-dependent locomotion of glial cells stops after exposure to cytochalasin B (Spooner *et al.*, 1971). Thus, the disappearance of the electron-dense band implies that this structure is composed of cytochalasin-sensitive microfilaments. If this were the case, then the bands might be conceived of as strips of microfilaments holding in alignment specialized membrane receptors responsible for the pairing of J-cilia. Such receptor-microfilament associations have previously been described (Brown and Revel, 1976). The problem with this hypothesis is that the electron-dense band disappears even if the ciliary junction is merely pulled apart. Thus, the possibility remains that, although the cytochalasins dissociate junctions, the disappearance of the band could be an indirect consequence of that dissociation.

The second, more gross, effect of all of the cytochalasins was the formation of tufts of ten to twenty J-cilia on the separated cilifers. Tufting may represent an increase in homotypic, as compared with heterotypic, adhesiveness leading to the breakage of the bonding between pairs of J-cilia. Since we have seen tufts on dissociating, but stll paired, cilifers, this remains a reasonable notion. However, the reverse possibility, that tufting is a consequence of dissociation or the disappearance of the electron-dense band, and only indirectly caused by cytochalasin, is not probable. That is, mechanical dissociation leads to the loss of the electron-dense band, but not to tufting. In fact, Murakami (1963) showed that the J-cilia become vigorously active when the ciliary junction is pulled apart.

The third effect of the cytochalasins, an increase in the number of hemocyanin-labeled Con A binding sites on the J-cilia, could come about in two ways. Cytochalasins could stimulate the production of new Con A binding sites, or they could modify the membrane, making extant, but unavailable, binding sites accessible to Con A. The disappearance of the electron-dense band could reflect a modification of the J-cilia, which might also expose or reactivate previously masked Con A receptors. The cytochalasins are known to affect the distribution and number of cell surface Con A receptors (binding sites) (Ash and Singer, 1976; Nicholson and Poste, 1976; Schlessenger *et al.*, 1976; Brown and Revel, 1976).

A fourth effect of the cytochalasins was the disruption of the microtubules in the J-cilia. However, only cytochalasins A and E had this effect, and only at relatively high concentration (15 $\mu$g/ml). Cytochalasin A has been shown to bind to tubulin and, in fact, to compete with colchicine for a binding site (Himes and Houston, 1976). However, the ciliary junction is disrupted by low doses of CCB (cytochalasin B) with no apparent effect on the microtubules in the J-cilia. We therefore conclude that the actions of CCA and CCE on microtubules are irrelevant to the adhesive mechanism responsible for the ciliary junctions.

### *Effects of concanavalin A*

Like the cytochalasins, Con A dissociates ciliary junctions and causes tufting of the J-cilia on the isolated cilifers. However, Con A-tufted cilia adhere at their tips, as do protozoan cilia or flagella after treatment with this lectin (see references in Introduction).

Presumably, Con A dissociates ciliary junctions by binding to receptors on the J-cilia membranes, thereby interfering with the molecular interaction between apposed ciliary pairs. We suggest tentatively, that the Con A receptors may be concentrated along the electron-dense band, held in this array by the presumed microfilamentous cytoskeleton of the band. Such an arrangement would explain the effectiveness of Con A in dissociating J-cilia. It might also explain, in part, the increase in Con A binding site labeling by cytochalasin, concomitant with the disappearance of the electron-dense band.

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# BIOCHEMICAL CHARACTERISTICS OF MACROURID FISHES DIFFERING IN THEIR DEPTHS OF DISTRIBUTION

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## ABSTRACT

Enzymic activities (units per gram wet weight of tissue) were measured in white skeletal muscle and brain tissue of five species of macrourid (rattail) fishes occurring over an approximately 5000 m depth gradient. Muscle protein and water contents were also determined. All species exhibited extremely low amounts of muscle enzymic activity for the glycolytic enzymes lactate dehydrogenase (LDH) and pyruvate kinase (PK), relative to values previously reported for shallow-living fishes. Malate dehydrogenase activity also was low, while citrate synthase (CS) activity was similar to levels found in shallow-living fishes. Interspecific differences among the rattails were large, especially for LDH activity which is a strong indicator of a fish's capacity for vigorous, burst swimming. *Coryphaenoides armatus*, a large rattail which is likely to be the most active swimmer among the species studied, had the highest enzymic activities and protein content, and, for LDH, PK, and CS, exhibited a significant scaling of enzymic activity with body mass. Scaling relationships were not observed for any other species. Brain enzymic activities were similar among all species. Muscle and brain enzymic activities also are reported for species belonging to four other deep-sea teleost families. The low levels of enzymes of energy metabolism found in skeletal muscle of these deep-sea fish species, and the interspecific variation in these activities are discussed in terms of the locomotory capacities and feeding strategies of these fishes. The potential usefulness of these types of enzyme data in estimating whole fish respiration rates is considered. We predict that the respiratory rates of the rattail species which have extremely low enzymic activity levels will be much lower than the respiratory rates previously measured for *C. armatus*.

## INTRODUCTION

The macrourid (rattail or grenadier) fishes comprise the dominant component of the bathyal fish fauna in many areas of the ocean (*e.g.*, Marshall, 1965, 1973; Iwamoto, 1970). There are some 300 macrourid species, a number of which may have cosmopolitan distributions (Marshall, 1973; Iwamoto and Stein, 1974). The rattails are an important component of deep-sea food webs (Haedrich and Henderson, 1974; Pearcy and Ambler, 1974). In the area south of New England, rattail fishes may account for up to 80 percent of the slope megafaunal biomass (Haedrich and Rowe, 1977; Haedrich *et al.*, 1980). Because of their feeding habits, the rattails

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Abbreviations: CS, citrate synthase; LDH, L-lactate dehydrogenase; MDH, L-malate dehydrogenase; PK, pyruvate kinase.

are important in terms of energy input and energy dispersal in the deep sea (Haedrich and Henderson, 1974; Pearcy and Ambler, 1974; McClellan, 1976), and may play an important role in the maintenance of macrofaunal species diversity (Dayton and Hessler, 1972; see Grassle and Sanders, 1973, for a contrasting view).

Rattail species differ in their feeding strategies and, even within a species, small and large individuals may differ in their prey and location in the water column. Some rattail species are motile scavengers, and have been observed to come to bait (Isaacs, 1969; Isaacs and Schwartzlose, 1975). Analyses of gut contents (Haedrich and Henderson, 1974; Pearcy and Ambler, 1974; McClellan, 1976) and head morphology (McClellan, 1976) have provided insight into their feeding habits and sources of food. Smaller rattail species, *e.g.*, *Nezumia bairdii, Coryphaenoides* (=*Lionurus*) *carapinus*, and smaller individuals of other species, *e.g.*, *C.* (=*Nematonurus*) *armatus* and *C. rupestris*, feed primarily on benthic or bottom-associated invertebrates (Haedrich and Henderson, 1974; Pearcy and Ambler, 1974; McClellan, 1976). Larger species, such as *C. rupestris, C. armatus*, and *C.* (=*Chalinura*) *leptolepis* may rely more on pelagic organisms, at least once these species reach a certain size (Haedrich and Henderson, 1974; Pearcy and Ambler, 1974; McClellan, 1976). It is not clear whether such pelagic prey are encountered near the bottom or much higher in the water column; nonetheless, it is likely that these rattails make excursions into midwater.

Rattail fishes have received relatively little physiological study, and we presently have few data concerning the physiological correlates of feeding and locomotory patterns. Smith and Hessler (1974) and Smith (1978) have determined the respiration rates of two large, common rattail species *in situ*. *Coryphaenoides acrolepis* was studied in the San Diego Trough at 1230 m; *C. armatus* was studied at 3650 m in the northwest Atlantic. Both species had very low respiration rates, consuming oxygen at only approximately 4 percent of the rates shown by similar-sized, shallow-living related species at the same experimental temperature. Both species fell on a similar weight *versus* respiration rate curve.

The present experiments were initiated to obtain additional information about the metabolic characteristics of rattail fishes, including data on interspecific differences in muscle metabolism that relate to variations in feeding strategy and locomotory capacity. Our approach involved measurement of the activities of key enzymes of energy metabolism (glycolysis and the citric acid cycle) in white skeletal muscle. Recent studies (Childress and Somero, 1979; Sullivan and Somero, 1980; Siebenaller and Somero, 1982; Somero, 1982) have demonstrated that the levels of activity of these enzymes in white muscle correlate strongly with the feeding strategy and capacity for vigorous swimming in a wide spectrum of marine fishes. Active pelagic swimmers like tunas have up to 1000-fold higher levels of glycolytic enzyme activity per gram wet weight of muscle than sluggish deep-sea species (Sullivan and Somero, 1980). Such enzymic indices are useful even in fine-scale comparisons of congeneric fishes which differ in their depth distributions (Siebenaller and Somero, 1982). Thus, a shallow-living scorpaenid fish, *Sebastolobus alascanus*, had approximately twice as much activity for several enzymes of energy metabolism in muscle as did a deep-living, closely related species, *S. altivelis*. Interspecific differences in muscle enzymic activity also correlate well with measured variations in oxygen consumption rate among midwater species (Childress and Somero, 1979), a finding which suggests that muscle enzymic activity data may be useful in making predictions about *in vivo* metabolic rates. Lastly, glycolytic enzymes of white skeletal muscle exhibit a striking scaling relationship with body size (Somero and Childress, 1980). Larger individuals of a species contain much higher levels of glycolytic enzymes per gram muscle than smaller individuals, a scaling function which appears to relate to the conservation of a stable capacity for burst locomotory performance in all sizes of individuals of a species (Somero and Childress, 1980). The presence of this type of metabolic scaling relationship, therefore, may provide some clue as to the importance of vigorous swimming activity in a species, and may indicate whether large and small members of a species have similar demands for intense locomotory performance.

Our comparisons of different-sized individuals of five macrourid species collected in the northwest Atlantic show that extremely large differences in muscle enzymic activity exist among species, and among different-sized individuals of the larger, more actively swimming species. However, there are no apparent differences in muscle enzymic activity among these species related to depth of occurrence *per se*. These data, plus observations made on several other deep-living fishes collected in the same trawls, are discussed in terms of interspecific differences in feeding behavior and metabolic requirements of life in the deep sea.

## Materials and Methods

### *Specimens*

Samples were taken with a 41-foot (12.5 m) Gulf of Mexico shrimp trawl, fished as in Haedrich *et al.* (1980), on cruise 93 of the R/V Oceanus in an area south of New England. Based on the distributional information described in Haedrich *et al.* (1980), samples were taken at appropriate depth intervals to obtain, at their depths of maximal abundance, the species used in this study. Sampling was conducted in late March and early April so that surface waters would be cold, and specimens would not be subjected to thermal shock. The fishes often had a heartbeat when brought to the surface, and were maintained in ice-cold seawater until frozen in a −80°C freezer at sea. Specimens were typically processed within an hour after the trawl was brought on deck. The samples were transported to the laboratory where they were maintained at −76°C.

A series of five macrourid species encompassing a depth range of 5000 m were obtained: *Nezumia bairdii*, *Coryphaenoides rupestris*, *Coryphaenoides* (=*Lionurus*) *carapinus*, *Coryphaenoides* (=*Nematonurus*) *armatus*, and *Coryphaenoides* (=*Chalinura*) *leptolepis*. The depth ranges and depths of maximal abundance of these species are reported in Table I. Specimens of the following deep-living species were also obtained and studied: *Halosauropsis macrochir* (Halosauridae), *Bathysaurus agassizi* (Bathysauridae), *Histiobranchus bathybius* (Synaphobranchidae), and *Dicrolene intranegra* (Brotulidae). The distributions of these species are given in Table III.

### *Enzymic activity determinations*

The fish were measured and weighed. Tissue samples were dissected from the frozen specimens and weighed, and the frozen tissue was added to an appropriate volume of 10 m*M* Tris-HCl buffer (pH 7.5 at 10°C). For white skeletal muscle, the dilution was either 4:1 (volume:weight) or 8:1, depending on the viscosity of the homogenate. For brain, the dilution was 8:1. Tissues were homogenized on ice in a ground glass tissue homogenizer (Kontes Glass Co., Duall-23 model). The homogenate was centrifuged at 2500 × *g* for 10 minutes at 4°C. The supernatant was used without further purification for enzymic activity measurements. All activities are expressed as μmoles substrate converted to product per minute per gram wet weight of tissue at 10°C.

TABLE I

*White skeletal muscle compositions and enzymic activity profiles of five macrourid fish species.*

| | N | Depth range* (m) | Depth of maximal abundance (m) | Mass [mean & range] (g) | % Water | Protein (mg/g) | Enzyme activity (units/g wet wt) [Mean ± S.D.] | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | LDH | PK | MDH | CS |
| *Nezumia bairdii* | 8 | 260–1965 | 600 | 54<br>24–102 | 81.2<br>± 1.2 (8) | 144.0<br>± 16.5 (4) | 6.9<br>± 2.7 | 4.6<br>± 2.2 | 17.5<br>± 10.1 | 0.62<br>± 0.15 |
| *Coryphaenoides rupestris* | 5 | 550–1960 | 1000 | 84<br>84–86 | 84.6<br>± 0.6 (4) | 142.1<br>± 31.0 (3) | 16.0<br>± 5.8 | 5.4<br>± 2.6 | 9.7<br>± 0.5 | 0.58<br>± 0.10 |
| *Coryphaenoides carapinus* | 11 | 1250–2740 | 2000 | 80<br>23–132 | 85.3<br>±0.8 (4) | 119.8<br>± 22.5 (4) | 4.7<br>± 2.4 | 5.9<br>± 2.2 | 6.8<br>± 0.9 | 0.50<br>± 0.19 |
| *Coryphaenoides armatus* | 13 | 1885–4815 | 2900 | 344<br>34–819 | 83.7<br>± 2.4 (9) | 177.1<br>± 18.2 (4) | 53.1<br>± 28.9 | 7.2<br>± 2.4 | 18.5<br>± 3.5 | 0.79<br>± 0.26 |
| *Coryphaenoides leptolepis* | 7 | 2288–4639 | 3500 | 456<br>90–960 | 82.3<br>± 0.5 (7) | 144.2<br>± 16.5 (4) | 4.3<br>± 1.2 | 2.6<br>± 0.3 | 6.9<br>± 1.0 | 0.41<br>± 0.14 |

* The depth ranges are from Haedrich *et al.*, 1980 and Haedrich, unpublished data.

The following enzymes were assayed in white skeletal muscle: L-lactate dehydrogenase (LDH, EC 1.1.1.27; L-lactate:$NAD^+$ oxidoreductase), pyruvate kinase (PK, EC 1.7.1.40; ATP: pyruvate phosphotransferase), L-malate dehydrogenase (MDH, EC 1.1.1.37; L-malate: $NAD^+$ oxidoreductase), and citrate synthase (CS, EC 4.1.3.7; citrate: oxaloacetate lyase (CoA-acetylating)). In brain tissue, LDH, PK, MDH and CS were assayed for some species. Assays were conducted as described in Somero and Childress (1980). For MDH appropriate controls were run to check for the decomposition of oxaloacetate during the course of the experiment.

### *Water and protein content of white muscle*

Wet weights were determined on muscle samples, and the samples were then dried at 60°C and weighed after 24 hours, when they had dried to a constant weight. The percentage water was determined from the difference between the initial wet weight and the final dry weight. Protein concentration of white muscle was determined using the microbiuret method of Itzhati and Gill (1964). Homogenates were prepared in distilled water and diluted to 100:1 (volume:weight) with NaOH to give a final NaOH concentration of 1 *M*. Samples were used without centrifugation. Protein concentration was determined, after addition of the biuret reagent, from the difference in absorbance at 310 and 390 nm, using bovine serum albumin as a standard.

## RESULTS

### *Macrourid white skeletal muscle*

The enzymic activities, and water and protein contents of the white skeletal muscle of the five macrourid species are given in Table I. As a group, these species display lower enzymic activity, lower protein content, and higher water content than do the shallower-living species which have been studied (*cf.* Childress and Somero, 1979; Sullivan and Somero, 1980). Lowered skeletal muscle enzymic activities have been observed for both midwater and benthopelagic fishes.

Within this group of rattails there is a wide variation of enzymic activity and protein content. This among-species variation is not correlated with depth of occurrence of the species. *Coryphaenoides armatus* displays strikingly higher levels of protein and enzymic activity per gram wet weight of muscle than do the other species. Also, for *C. armatus*, there is a statistically significant scaling of enzymic activity to body mass for CS, PK, and LDH (Fig. 1). The equations for these scaling relationships are: $A = 1.0\ W^{-0.59 \pm 0.005}$ for CS; $A = 1.83\ W^{0.24 \pm 0.13}$ for PK, and $A = 1.16\ W^{0.66 \pm 0.20}$ for LDH. The 95% confidence intervals are given for the

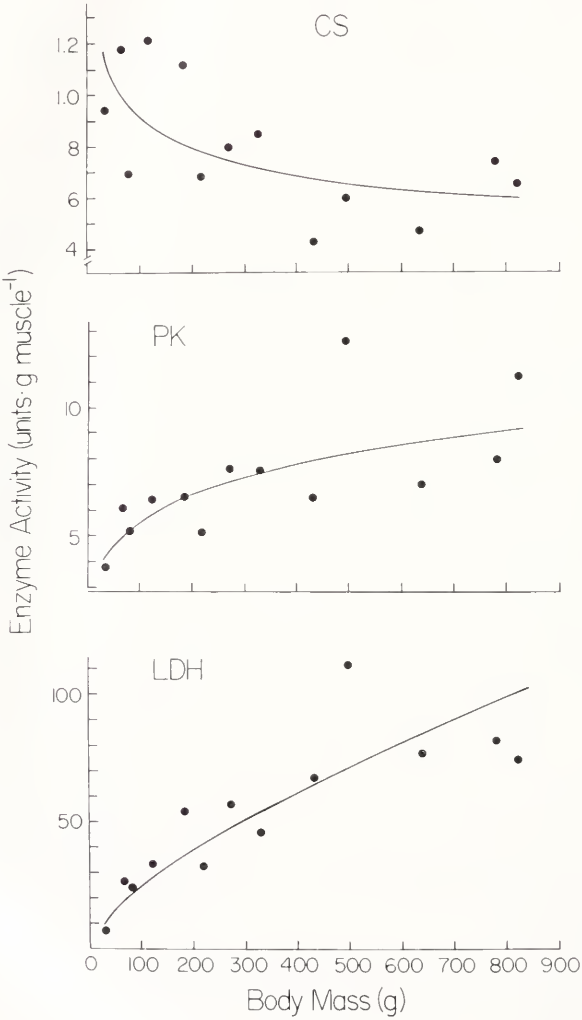

FIGURE 1. The scaling of enzymic activity in white skeletal muscle *versus* body mass for individuals of *Coryphaenoides armatus*. Citrate synthase (CS), pyruvate kinase (PK) and lactate dehydrogenase (LDH) displayed statistically significant scaling of activity *versus* body mass. The equations fitting these data are given in Results; the lines shown were fit by these equations.

Table II

*Enzymic activity in brain tissue of three species of* Coryphaenoides.

| | Mass [mean & range] (g) | Enzyme activity (units/g wet wt) [Mean± S.D.] | | | |
|---|---|---|---|---|---|
| | | LDH | PK | MDH | CS |
| *Coryphaenoides rupestris* | 66<br>58–86 | 27.8<br>± 4.3 | 22.0<br>± 6.7 | 43.5<br>± 9.1 | 2.0<br>± 0.2 |
| *Coryphaenoides armatus* | 255<br>67–494 | 22.0<br>± 3.7 | 13.3<br>± 1.2 | 50.7<br>± 5.5 | 1.4<br>± 0.2 |
| *Coryphaenoides leptolepis* | 625<br>278–960 | 17.6<br>± 2.6 | 13.8<br>± 1.0 | 35.1<br>± 4.2 | 1.3<br>± 0.2 |

Four individuals of each species were used.

scaling exponents. "A" is the enzymic activity and "W" the wet weight of the entire fish in grams. None of the other macrourids showed detectable mass-related scaling of enzymic activity. For example, *C. leptolepis*, for which we had individuals ranging in mass from 90 to 960 grams, had a range of LDH activity of only 2.3 to 5.3 units per gram wet weight, with no size-related variation. The scaling patterns observed for white muscle enzymes of *C. armatus* agree with those noted for a variety of shallow-living fishes (Somero and Childress, 1980) in that the activities of the two glycolytic enzymes, LDH and PK, increase with rising body mass, while the activity of the citric acid cycle (=aerobically poised) enzyme CS displays lower activity per gram muscle in larger specimens.

### *Macrourid brain tissue*

The activities of the four enzymes assayed in skeletal muscle also were measured in brain tissue of *C. rupestris*, *C. leptolepis*, and *C. armatus* (Table II). The values are somewhat variable, but generally similar among the three species. These activities are comparable to those reported for other fishes, both shallow- and deep-living (Childress and Somero, 1979; Sullivan and Somero, 1980; Siebenaller and Somero, 1982). We observed no scaling relationships for the brain enzymes, but this result may be due to the small sample size used in the study.

### *Muscle enzymic activities and compositions of other deep-sea families*

The enzymic activities and water and protein contents of white skeletal muscle of representatives of four other deep-sea fish families are given in Table III. The enzymic activities in these species are low and within the range found for the macrourid species.

There is variation among these species, and wide variation between individuals of *Histiobranchus bathybius*. The protein and water contents of the muscle of this species were extremely variable, and some component of the tissue may have caused interference with the protein measurements. These data are not reported here.

The number of individuals and the size range of individuals which were taken in our sampling program are not adequate to permit us to address the question of mass-related scaling in these species.

TABLE III

*White skeletal muscle compositions and enzymic activity profiles for species of four deep-living fish families.*

| | N | Depth range* (m) | Depth of maximal abundance (m) | Mass [mean & range] (g) | % Water | Protein (mg/g) | Enzyme activity (units/g wet wt) [Mean ± S.D.] | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | LDH | MDH | PK | CS |
| *Halosauropsis macrochir* | 3 | 1500–5179 | 2300 | 290<br>248–365 | 81.2<br>± 0.4 | 90.7<br>± 24.4 | 11.6<br>± 1.7 | 3.6<br>± 0.03 | 2.2<br>± 0.4 | 0.40<br>± 0.75 |
| *Bathysaurus agassizi* | 2 | 1500–2967 | 2000 | 625<br>433–817 | 80.9<br>± 0.4 | 107.8<br>± 11.0 | 35.4<br>± 6.0 | 9.2<br>± 1.8 | 11.0<br>± 0.9 | 0.81<br>± 0.21 |
| *Histiobranchus bathybius* | 2 | 1885–1093 | 2900 | 793<br>328–1258 | — | — | 53.0<br>± 59.8 | 8.5<br>± 7.8 | 12.3<br>± 12.6 | 0.61<br>± 0.52 |
| *Dichrolene intranegra* | 1 | 720–1960 | 1000 | 85 | 81.1 | 100.5 | 46.4 | 13.2 | 6.4 | 1.21 |

* Depth ranges are from Haedrich *et al.*, 1980, and Haedrich, unpublished data.

## DISCUSSION

All of the species examined in this study have extremely low levels of LDH, PK, and MDH activity per gram of skeletal muscle compared to shallow-living fishes. For example, activities of LDH, the enzyme which appears to be the best index of a fish's capacity for intense, burst swimming (Somero and Childress, 1980), range between approximately 200 and 1000 units per gram in muscle of shallow-living, pelagic fishes; and 4 to 150 units per gram in deep-living fishes (Sullivan and Somero, 1980; Tables I and III). Citrate synthase, an indicator enzyme of the citric acid cycle, is present in only low activities in white muscle, a reflection of the anaerobic poise of this tissue (*cf.* Somero and Childress, 1980). CS activity varies only slightly among species, and only a small reduction in CS activity is noted in deeper-living fishes (Sullivan and Somero, 1980). MDH activity is intermediate between the two glycolytic enzymes (LDH and PK) and CS in terms of interspecific variation. MDH may play some role in cytoplasmic redox balance, albeit LDH is the dominant factor in this context, and it may also contribute to the shuttling of reducing equivalents between the cytosol and the mitochondria, and to the function of the citric acid cycle. Because of this variety of roles, MDH is less apt to be a strong indicator of burst swimming capacity than either LDH or PK. The results of the present study, like those of earlier comparisons of enzymic activities (Childress and Somero, 1979; Sullivan and Somero, 1980; and Siebenaller and Somero, 1982), indicate that reduction in the capacity for anaerobic glycolysis in muscle, *i.e.*, in burst swimming ability, is a major feature of adaptation to life in the deep sea.

There is wide variation of glycolytic activity in white muscle among the five rattail species, however, especially in the case of LDH. The highest levels of enzymic activity, and the only significant scaling relationships between enzymic activity and body mass, are found for *C. armatus* (Table I; Figure 1). At least larger-sized individuals of this species appear to make excursions into midwater to prey on pelagic organisms (Haedrich and Henderson, 1974; Pearcy and Ambler, 1974). The relatively high levels of glycolytic enzymes in white muscle of *C. armatus*, and the body-mass-related scaling noted for LDH and PK, may be reflections of a relatively high capacity for swimming compared to the other rattail species we examined. *Coryphaenoides armatus* also had the highest muscle protein content of all the rattails studied.

The second-highest levels of LDH were found in *C. rupestris*. This species has a poorly ossified skeleton and weak musculature development (Marshall, 1973), but it has been reported to make excursions into the water column, and to feed on pelagic prey (Haedrich, 1974). *Nezumia bairdii* and *C. carapinus* feed on benthic invertebrates (Haedrich and Henderson, 1974; Pearcy and Ambler, 1974; McClellan, 1976). Although larger individuals of *C. leptolepis* may take pelagic prey (Pearcy and Ambler, 1974), this species has a poorly developed swimbladder and probably stays near the bottom (Marshall, 1973; Pearcy and Ambler, 1974). These species have very low levels of glycolytic enzymic activity in their white skeletal muscle, which may reflect a low potential for burst swimming correlated with this foraging habit.

The low levels of enzymic activity found in *C. leptolepis* relative to *C. armatus* demonstrate that body size does not contribute significantly to the interspecific differences noted in enzymic activity. Thus, large individuals of *C. armatus* had approximately ten times as much LDH activity and three times as much PK activity as similar-sized individuals of *C. leptolepis*. The finding that skeletal muscle LDH activity is low and very similar in all sizes of *C. leptolepis* examined indicates that this fish is unlikely to have much capacity for rapid burst locomotory activity. Burst swimming capacity would, in fact, decrease considerably with increasing body size for *C. leptolepis*, since a scaling relationship between body mass and LDH activity comparable to that found for *C. armatus* muscle is needed to conserve a constant burst swimming capacity as body size increases (Somero and Childress, 1980).

Smith (1978) measured respiration rates in *C. armatus* that were very low in comparison to those of shallow-living fishes at similar temperatures. Previously, Smith and Hessler (1974) measured a comparably low respiration rate for *C. acrolepis*. The respiration rate of *C. armatus* scaled with body mass according to the equation: $Y = 0.03\ W^{0.65}$, where Y is the oxygen consumption rate (ml/h) and W is the wet weight of the fish (g). The scaling we have determined for LDH activity in skeletal muscle of *C. armatus* is fit by a similar power function: $A = 1.16\ W^{0.66}$, where A is the LDH activity (units per g wet weight) and W is fish wet weight (g). The virtually identical scaling exponents indicate a linear relationship between oxygen consumption rate and LDH activity in this species.

Childress and Somero (1979) demonstrated an interspecific correlation between LDH activity and oxygen consumption rate for midwater fishes. A similar relationship of oxygen consumption rate and LDH activity for benthopelagic rattails is suggested by the scaling relationships for oxygen consumption and LDH activity of *C. armatus*, as discussed above, and the finding of similar levels of LDH activity in *C. acrolepis* and *C. armatus* (Sullivan and Somero, 1980; Table I). The *in situ* respiration rates of *C. acrolepis* and *C. armatus* were also similar (Smith and Hessler, 1974; Smith, 1978). However, the relationship of LDH activity and respiration in macrourids does not fall on the same curve as the data for midwater fishes. Also, for the midwater fishes examined by Childress and Somero (1979), MDH activity correlated with oxygen consumption rates. MDH activity in *C. armatus* does not scale as the same fractional exponent of mass as does oxygen consumption, and thus may not be a predictor of respiration rate in macrourid species.

Assuming a relationship between respiration rate and LDH activity in rattails, the very low activities of LDH observed in *N. bairdii*, *C. rupestris*, *C. carapinus*, and *C. leptolepis* are indicative of extremely low rates of oxygen consumption.

These four rattail species may have some of the lowest metabolic rates of any fishes. The body mass *versus* respiration rate relationship described by Smith (1978) for *C. acrolepis* and *C. armatus* may, therefore, overestimate the oxygen consumption rates of the other rattail species we have studied.

Despite the wide interspecific variation in the activities of skeletal muscle enzymes, relatively small interspecific differences were found in comparisons of brain enzymes (Table II). This finding agrees with previous reports of Childress and Somero (1979), Sullivan and Somero (1980) and Siebenaller and Somero (1982), who found no evidence for depth- or activity-related trends in brain enzymic activity. The general similarity in brain enzymic activities for both glycolytic and citric acid cycle enzymes among widely different fishes from shallow and deep-sea habitats suggests that the requirements of neural function are similar among different fishes.

The muscle enzymic activities of the representatives of the four other deep-sea fish families also are very low relative to shallow-living, actively swimming fishes (Table III; Sullivan and Somero, 1980). These low activities are again likely to be a reflection of a relatively low capacity for active swimming. Marshall (1973) considers the rattails, halosaurs and brotulids to be slow, intermittent swimmers. The low muscle enzymic activities found in *H. macrochir* and *D. intranegra* and the smaller rattails are consistent with this view. The low skeletal muscle activities of the bathysaur, *B. agassizi*, also suggest a similar locomotory capacity.

A high amount of variation between individuals was noted for the synaphobranchid fish, *Histiobranchus bathybius*. The wide variation in muscle enzymic activities could be a reflection of a strong scaling relationship like that noted for *C. armatus*; however, we captured too few specimens of *H. bathybius* to test this hypothesis. The finding that LDH activity in muscle of *H. bathybius* reached 95 units per gram in the larger specimen examined (mass = 1258 g) and 156 units per gram in a specimen studied by Sullivan and Somero (1980) (mass not known due to lack of a complete specimen), suggests that this species is capable of an active locomotory style.

In summary, our examination of five rattail fishes has revealed that depth of occurrence *per se* is not a factor in the differences in white muscle enzymic activities within this family of fishes. Rather, the differences in muscle enzymic activities appear to reflect interspecific variation in feeding habits. The large rattail, *C. armatus*, possessed the highest levels of glycolytic enzymes and the only scaling of these activities with body mass. Both traits are argued to be evidence for an active locomotory habit, at least relative to other rattail fishes. The extremely low muscle enzymic activities found in the species we examined are taken as evidence for very low whole organism respiration rates of these fishes. To the extent that whole organism oxygen consumption rate is linearly related to LDH activity of muscle (Childress and Somero, 1979), we propose that the four rattail species found to contain the lowest LDH activities have extraordinarily low respiratory rates, rates that are considerably lower than those which would be predicted by extrapolation using the respiration rate *versus* body mass relationship developed by Smith (1978) in his studies of *C. armatus*.

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# ADAPTIVE SIGNIFICANCE OF SEMILUNAR CYCLES OF LARVAL RELEASE IN FIDDLER CRABS (GENUS *UCA*): TEST OF AN HYPOTHESIS

JOHN H. CHRISTY

*Belle W. Baruch Institute for Marine Biology and Coastal Research, University of South Carolina, Columbia, SC 29208*

## ABSTRACT

The hypothesis that semilunar timing of larval release by fiddler crabs (genus *Uca*) results in transport of the final larval stage (megalopa) by spring tide currents to substrates in the upper estuary occupied by adults was tested and rejected. Water temperatures in the North Inlet estuary, South Carolina, increased from approximately 20°C to 28°C and the length of larval life decreased during the May–September breeding season. Nevertheless, ovigerous female *U. pugilator, U. pugnax,* and *U. minax*, collected bimonthly and maintained in the laboratory, released larvae ±1.5 d of the full and new moons throughout most of the breeding season. Megalopae of *Uca* spp. were most abundant in a small tidal creek in the upper estuary during nocturnal flood tides and near the bottom about 5 d before and after the spring tides in September. *Uca* spp. and several other estuarine crabs appear to release larvae near the times of the high tides that are followed by the nocturnal ebb tides of greatest amplitude during the semilunar cycle. At North Inlet, such timing results in rapid seaward transport of newly hatched zoeae and subsequent export into coastal waters. Convergence among estuarine brachyurans in the timing of larval release probably reflects a shared adaptive response to selective factors, such as lethal combinations of high temperatures and low salinities, or predation by diurnal planktivors, that cause high larval mortality during the day in the upper estuary.

## INTRODUCTION

Semilunar cycles of larval release have been reported for several estuarine crabs (Christy and Stancyk, 1982). Such cycles can be inferred for 17 species of fiddler crabs (genus *Uca*) from cycles of male courtship activity (Crane, 1958; von Hagen, 1970; Christy, 1978; Zucker, 1978) female sexual receptivity, mate choice, and incubation behavior (Christy, 1978), ovarian and egg development (von Hagen, 1962, 1970; Feest, 1969; Zucker, 1973; DeCoursey, 1981) and variation in the density of newly hatched zoeae in the plankton (Christy and Stancyk, 1982). Direct evidence of a semilunar hatching rhythm has been obtained for *U. pugnax* under laboratory conditions (Wheeler, 1978) and for *U. pugilator* in both the laboratory and the field (DeCoursey, 1981). Tidal and diel timing of larval release has been well described for *Uca pugilator* (DeCoursey, 1979, 1981; Bergin, 1981), *U. pugnax*, and *U. minax* (DeCoursey, 1979); all three species release larvae at night near the time of high tide.

Hypotheses concerning the ecological consequences and the adaptive significance of semilunar cycles of larval release in *Uca* spp. and other estuarine crabs fall into two classes: those that invoke semilunar variation in factors such as food avail-

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ability, susceptibility to predation, and tidal exposure that may effect the reproductive success of adult males and females (Bergin, 1978, 1981; Zucker, 1978; DeCoursey, 1979) and those that rely on semilunar variation in factors that effect the dispersal, survival, and settlement rates of larvae (von Hagen, 1970; Bergin, 1978; Christy, 1978; Wheeler, 1978; Zucker, 1978; DeCoursey, 1979; Saigusa, 1981). Although supporting evidence for some of these hypotheses has been sought in interspecific comparisons of adult (Zucker, 1978) and larval (Saigusa, 1981) ecology, no hypothesis has been critically tested.

This paper presents a test of the hypothesis that the timing of larval release by female fiddler crabs results in transport of the final larval stage (megalopa) by spring tide currents to substrates suitable for settlement (Christy, 1978). This hypothesis is based on the following observations and argument. Vertical migration within or between larval stages relative to tidal and residual currents may aid retention of crab larvae in estuaries (Sandifer, 1975; Cronin, 1979). However, *Uca* megalopae have been found tens of kilometers from habitats occupied by adults in stratified estuaries where larval vertical migration should be most effective in reducing seaward transport (Dudley and Judy, 1971; Sandifer, 1973). To return to adult habitats, which extend commonly to the heads (*sensu* Carriker, 1967) of estuaries (Crane, 1975), megalopae often must move many kilometers up-estuary before they settle and molt to crabs. They might do this by remaining on or near the bottom during ebb tides where currents are weak, then rising in the water column into stronger currents during flood tides (Carriker, 1967; Christy, 1978). Given these patterns of transport and behavior, megalopae that could settle during spring tides, when current velocities are at a maximum, would return to adult habitats faster than those that were ready to settle at other times. If larval mortality is proportional to the time spent in the water column (Thorson, 1946, 1950; Vance, 1973), more megalopae that were ready to settle during spring tides would reach adult habitats than those moving into the upper estuary at other times; selection might favor females that release zoeae that become megalopae during spring tides. This idea appears consistent with the timing of larval release by *U. pugilator* on the southwest coast of Florida (Christy, 1978).

The hypothesis requires that the time between larval release and a spring tide must equal an integral multiple of the length of larval development. Development rates of brachyuran larvae depend strongly on temperature (*e.g.*, Costlow *et al.*, 1960, 1962, 1966; Vernberg and Vernberg, 1975). On the east coast of the United States *Uca* spp. begin breeding when water temperatures are cool and end breeding when temperatures are considerably higher (Crane, 1943). If the hypothesis is correct, and if larval development rates are temperature dependent in the field, then there should be a change in the phase relationship between the semilunar cycles of larval release and the spring tides as water temperature increases seasonally, and megalopae should be transported to adult habitats only during spring tides. Therefore, the hypothesis was tested by monitoring when female *Uca* spp. release larvae throughout a breeding season and by determining when megalopae colonize adult habitats.

## MATERIALS AND METHODS

This study was conducted at the North Inlet estuary and the Field Laboratory of the Belle W. Baruch Institute for Marine Biology and Coastal Research, Georgetown County, South Carolina (Fig. 1). This is a high salinity, homogeneously mixed estuary in which the currents are dominated by the semidiurnal partial tide (Kjerfve and Proehl, 1979).

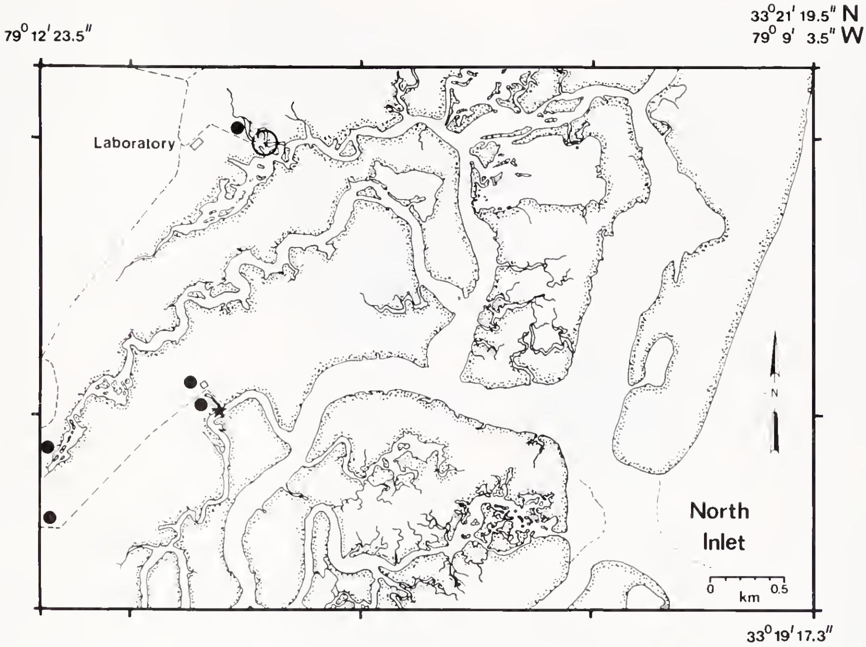

FIGURE 1. Northern portion of the North Inlet estuary. Solid circles indicate the sites where ovigerous female *Uca* were collected. The open circle shows the site where the plankton was sampled for crab megalopae. The star shows the location of the tide gauge. The approximate boundaries of the flood and ebb deltas are indicated by the dashed lines. (After the North Inlet quadrangle map, United States Geological Survey, 1942. Inlet morphology is approximate.)

### *Larval release in the laboratory*

Cycles of larval release were determined by maintaining sequential collections of ovigerous females under controlled conditions and counting daily the number that released larvae. Ovigerous female *U. pugilator* and *U. pugnax* were dug from intertidal substrates (Fig. 1) during low tide at approximately 2-week intervals from 28 July to 12 September 1978, and from 4 May to 18 September 1979. Ovigerous female *U. minax* were collected concurrently with the other species in 1978 only. Approximately 100 females of each species were collected in each sample. Female crabs were maintained in the dark in incubation tubes suspended in filtered sea water (1 μm, 34‰ salinity) in an insulated fiberglass tank supplied with four undergravel filters, two 500-watt immersion heaters controlled by a thermoregulator, and an opaque lid. The incubation tubes allowed females to rest in about 0.5 cm of water and retained larvae after hatching (Fig. 2). From 28 July to 22 September 1978 the temperature in the tank was 28°C. During 1979, the tank temperature was 24°C from 4 to 31 May, 26°C from 1 June to 29 July, and 28°C from 30 July to 2 October. These temperatures corresponded closely to the substrate temperatures at the depths at which females were collected.

Each morning the number of females of each species that released larvae was recorded and assigned to the date of the previous night. Every female released all her larvae in a single night. The criteria for scoring release were the absence of eggs on the pleopods of females and the presence of zoeae in the incubation tubes. The

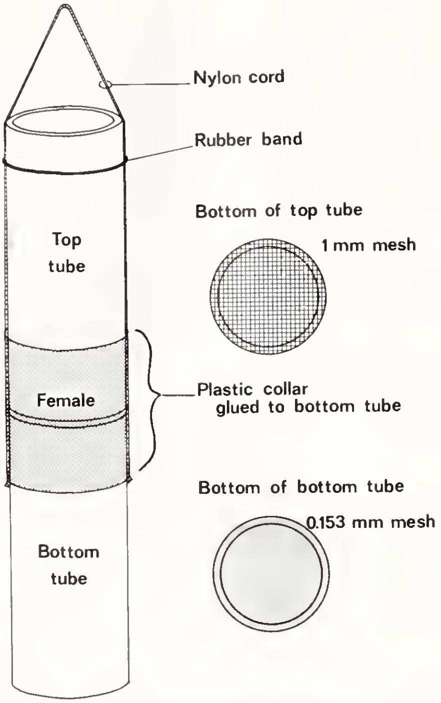

FIGURE 2. Incubation tube used to house ovigerous female *Uca* in a temperature-controlled seawater tank.

latter criterion was necessary because females occasionally, though rarely (5 out of 3,015 females), ate their eggs before they hatched.

### *Larval development rates*

To test the assumption that larval development rates depend on temperature, *U. pugilator* larvae were reared at 22 and 28°C. About 1 h after hatching, zoeae obtained from females in the incubation tubes were mixed in a glass bowl, concentrated in a light beam, and transferred by pipette into filtered sea water (0.45 $\mu$m, 34‰ salinity) in the compartments of plastic boxes fitted with hinged lids. Ten zoeae were placed into 100 ml of water in each compartment. Zoeae were transferred daily to fresh seawater in clean compartments and fed a surfeit of 1- to 3-h old *Artemia salina* nauplii. All zoeae were reared under a 14L:10D cycle in an environmental chamber. The 22°C experiment was begun on the night of 11 May 1979 with 230 zoeae from 9 females. The 28°C experiment was begun on 2 September 1978 with 160 zoeae from 12 females. Zoeae from several females were pooled so that estimates of development rates would include components of variation due to differences among broods. The water temperatures at high tide in the marsh matched the rearing temperatures on the dates the experiments were begun.

### *Plankton samples*

To determine when megalopae reach the upper estuary, plankton samples were collected during day and night flood tides at a single station near the head of a small tidal creek (1–1.5 m deep at mid-tide) (Fig. 1). All three species of *Uca* occur abundantly on the creek banks and in the marsh adjacent to the sampling site. Samples were taken by hand from a foot bridge with a conical net (0.5 by 2 m, 800-$\mu$m mesh Nitex cloth) fitted with a flowmeter.

From 31 August to 7 September 1978, two to four 5-min samples were taken at 10-min intervals beginning at mid-flood tide (3 h after slack low water). For the first 2.5 min of each sample, the net was suspended just above the creek bottom; for the remaining 2.5 min, the net was raised so that it was just under the surface of the water. From 7 to 11 September, two separate 5-min top and bottom samples were taken at each mid-flood tide. From 12 to 23 September, one top and one bottom sample were taken. Finally, on 20 and 21 September, one top and one bottom sample were taken during mid-ebb tide (3 h after slack high water) at night. From 10 to 25 $m^3$ (mean $\pm$ SD = $15.44 \pm 4.90$ $m^3$) of water was filtered during each 5-min sample. All *Uca* megalopae were counted in each sample. Megalopal densities are the means of the densities of the sequential samples collected during each sampling period.

### *Physical measurements*

From 19 July to 4 October 1978 and from 11 January to 23 July 1979 surface water temperatures were measured within 1 h of the time of a high tide at or near the site where the plankton samples were taken. From 11 January to 30 April 1979 water temperatures were recorded during the day. At all other times temperatures were taken at night. On two occasions temperatures were measured within 30 min of slack high water at several points along a transect from the mouth of North Inlet to the upper marsh and were found to vary less than 1.5°C.

Tide heights and amplitudes were obtained from a tide gauge located in the North Inlet estuary (Fig. 1). This paper relates semilunar cycles of larval release to semilunar cycles in the amplitude of nocturnal ebb tides. A nocturnal ebb tide is defined as one that follows a high tide that occurs between the hours of sunset and sunrise. When both high tides occurred during daylight, the one closest to sunrise or sunset was designated as the nocturnal high tide for that day.

## RESULTS

### *Breeding seasonality and water temperatures*

Crab activity was observed daily during low tide throughout the 1979 breeding season. Male *U. pugilator* first courted on 13 March. By the end of March, both *U. pugilator* and *U. pugnax* were courting, and both species fed during low tide in aggregations in the lower intertidal zone. Courtship activity declined rapidly after the full moon on 16 September 1978 and after the new moon on 21 September 1979, and ended for the year about 5 d after both dates.

Water temperature increased rapidly in the spring from a low of about 7°C in February to about 20°C by late April and early May. Temperatures continued to rise to about 28°C in the late summer (Fig. 3).

The length of incubation in summer for several species of *Uca* is from 12 to 15 d (Feest, 1969; von Hagen, 1970; Greenspan, 1975; Christy, 1978). If females mated and began incubation in late March and cool spring temperatures no more than double the period of incubation, then females may have first released larvae in late April. Larval release by *U. pugilator* first occurred during the period of 2 to 6 May in 1981 (J. Christy, unpublished). Stage I *Uca* zoeae were common in plankton

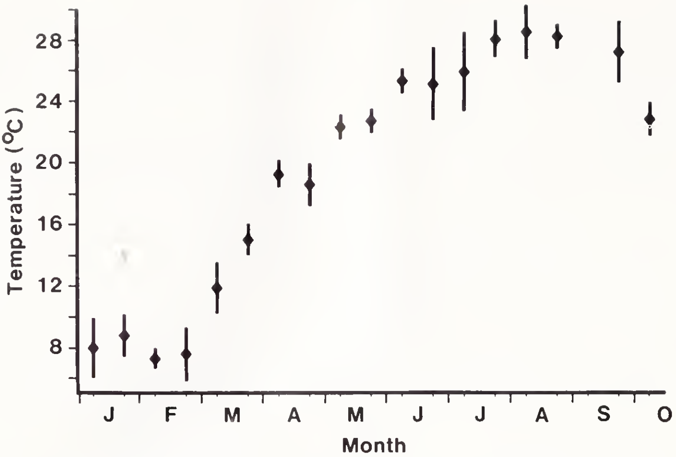

FIGURE 3. Bimonthly means of daily water temperatures in the North Inlet estuary recorded within 1 h of diurnal (11 January–30 April) or nocturnal (1 May–4 October) high tides. Temperatures were recorded in 1978 (19 July–4 October) and in 1979 (11 January–23 July). The bars indicate two standard errors above and below each mean.

samples taken at the study site in mid-May, 1979 (Christy and Stancyk, 1982). At the beginning of the breeding season, *Uca* larvae probably develop in water averaging 20 to 22°C.

### *Larval development rates*

Development of *U. pugilator* zoeae was significantly slower at 22°C (mean ± SD = 19.4 ± 2.47 d, range = 16–27 d, $N = 230$, 76% survival to megalopa) than at 28°C (mean ± SD = 14.7 ± 2.39 d, range = 12–20 d, $N = 160$, 59% survival to megalopa) ($t_{267} = 19.013$, $P \ll 0.001$). These experiments confirm that development rates of *Uca* larvae are temperature dependent in the laboratory (Vernberg and Vernberg, 1975). On the assumption that larval development rates also depend on temperature in the field, it seems justified to expect, if the hypothesis is correct, a seasonal change in the timing of larval release relative to the spring tides as water temperatures increase during the breeding season.

### *Larval release in the laboratory*

During August and September 1978, *U. pugilator, U. pugnax,* and *U. minax* displayed marked semilunar cycles of larval release (Fig. 4) as did *U. pugilator* and *U. pugnax* during most of the 1979 breeding season (Fig. 5). Females of all three species released larvae (mean ± SD) 0.06 ± 1.116 d before the date of a full or new moon (Table I). In 1978 the average deviations of the mean dates of release from the dates of the syzygies were 0.24 d for *U. pugilator,* −0.09 d for *U. pugnax,* and −0.80 d for *U. minax.* In 1979 *U. pugilator* released larvae, on average, 0.19 d and *U. pugnax* 0.41 d after a full or new moon. There was no significant correlation between the mean date of larval release for each cycle relative to the date of the full or new moon and the sequential rank of the date of each syzygy during the breeding season for either *U. pugilator* [Kendall's coefficient of rank correlation (Sokal and Rohlf, 1969); $\tau = 0.36$, $N = 8$, $P > 0.05$] or *U. pugnax* ($\tau = 0.27$, $N = 8$, $P > 0.05$). The expected seasonal change in the timing of larval release relative to the full and new moons and spring tides did not occur.

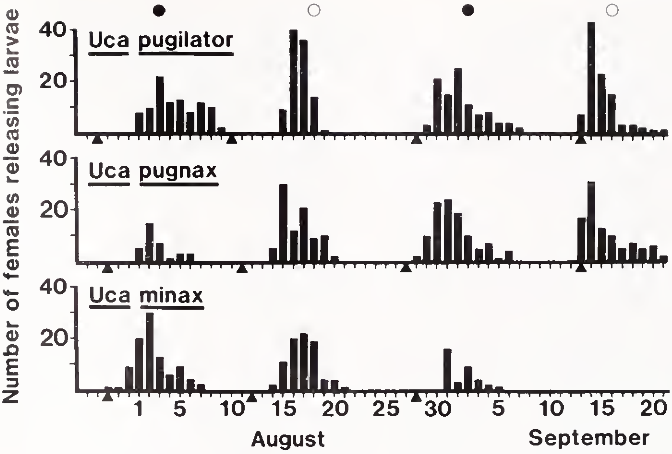

FIGURE 4. Cycles of larval release by *Uca* spp. in the laboratory in 1978. The triangles show the dates on which each sample of ovigerous females was collected. The dates of the full and new moons are indicated by the open and solid circles, respectively.

### *Variation in the density of* Uca *megalopae*

*Uca* megalopae were significantly more abundant in the water column during the night than during the day [Fig. 6; Wilcoxon's signed ranks test comparing the

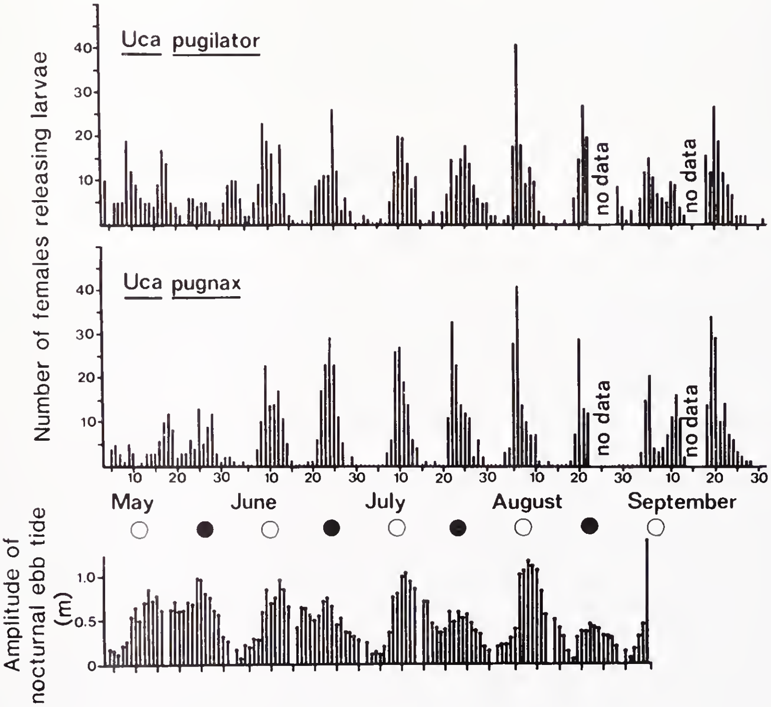

FIGURE 5. Cycles of larval release by *Uca* spp. in the laboratory in 1979 and the amplitudes of the nocturnal ebb tides at North Inlet. Dots on the x-axis indicate that ovigerous females were present in the laboratory but none released larvae. Moon phases are indicated as in Figure 4.

TABLE I

*Deviation of the mean date (±SD, days) of larval release in the laboratory from the date of a full or new moon.*

| Date of syzygy | *U. pugilator* | | *U. pugnax* | | *U. minax* | |
|---|---|---|---|---|---|---|
| | Mean date | *N* | Mean date | *N* | Mean date | *N* |
| 1978 | | | | | | |
| Aug. 3 | 1.40 ± 2.29 | 99 | −0.26 ± 1.46 | 34 | −0.65 ± 1.75 | 95 |
| Aug. 18 | −1.42 ± 6.90 | 100 | −1.58 ± 1.60 | 89 | −1.06 ± 1.46 | 83 |
| Sept. 2 | −0.69 ± 2.20 | 100 | −1.43 ± 2.10 | 100 | −0.69 ± 1.48 | 35 |
| 1979 | | | | | | |
| May 11 | −0.38 ± 4.28 | 99 | 1.82 ± 5.21 | 51 | | |
| May 25 | −0.30 ± 6.80 | 100 | −1.29 ± 4.26 | 82 | | |
| June 10 | −0.62 ± 2.07 | 110 | 0.12 ± 2.53 | 102 | | |
| June 24 | −0.54 ± 3.20 | 100 | −0.37 ± 2.06 | 116 | | |
| July 9 | 1.41 ± 2.95 | 97 | 1.24 ± 2.40 | 109 | | |
| July 23 | 2.27 ± 7.08 | 115 | 0.57 ± 2.03 | 114 | | |
| Aug. 7 | 0.06 ± 1.96 | 118 | −0.63 ± 1.61 | 115 | | |
| Sept. 6 | −0.20 ± 3.98 | 104 | 1.84 ± 3.14 | 96 | | |

density of megalopae in each nocturnal flood tide and subsequent diurnal flood tide (Sokal and Rohlf, 1969); $T = 11$, $N = 17$, $P < 0.005$] and significantly more (80% on average) were moving in the lower 50 cm of the water column at mid-flood tide than near the surface (Wilcoxon's signed rank test comparing densities each night in surface and bottom samples; $T = 50$, $N = 38$, $P < 0.005$). On 19 and 20 September densities of megalopae were 2.02 and 3.31 per $m^3$ during nocturnal flood tides. In contrast, approximately 6 h later during nocturnal ebb tides, densities had dropped to 0.09 and 0.02 megalopae per $m^3$ on the two nights, respectively. Megalopae were most abundant about 5 d before and after the spring tides (Fig. 6), not during the spring tides as would be expected if the hypothesis were correct.

## DISCUSSION

Semilunar cycles of larval release by *U. pugilator* and *U. pugnax* in the laboratory in 1979 corresponded closely to semilunar cycles in the density of newly hatched stage I zoeae in the upper estuary (Christy and Stancyk, 1982 This indicates that the timing of larval release in the laboratory probably accurately estimates the timing of larval release in the field.

Neither *U. pugilator* nor *U. pugnax* exhibited a semilunar cycle of larval release during May 1979. Wheeler (1978) reported a similar aperiodicity in June for *U. pugnax* collected in Delaware and maintained in the laboratory. *Sesarma cinereum* from North Inlet also lacked cycles of reproduction and hatching in the field and laboratory during May and June in both 1978 and 1979, though this crab exhibited marked semilunar cycles of larval release at other times (Dollard, 1980). The causes and consequences of aperiodic larval release by these crabs in the early breeding season are unknown.

The timing of larval release by *U. pugilator* and *U. pugnax* changed little during the 1979 breeding season even though water temperatures increased at least 5°C. The rearing experiments confirmed that the length of larval development decreases with an increase in temperature. If the larval lifespan in the field decreased as the water temperature increased from May to September, then it is clear that females

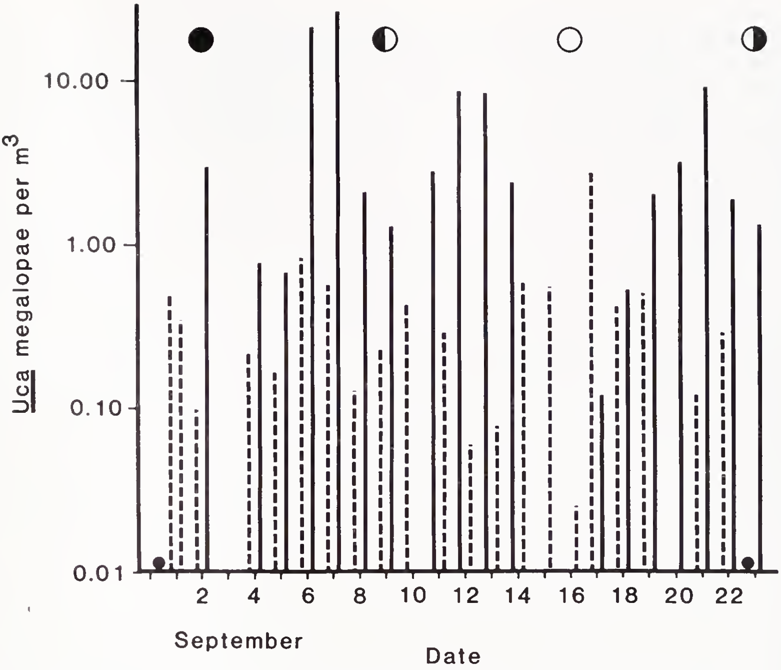

FIGURE 6. Temporal variation in the density of *Uca* spp. megalopae during September 1978. Samples were collected during daytime (broken bars) and nighttime (solid bars) flood tides in a tidal creek in the upper estuary (Fig. 1). Densities of 0 megalopae per $m^3$ are indicated by dots on the x-axis. Full and new moons are indicated as in Figure 4. First and last quarter moons are indicated by half-solid circles.

did not time release so that megalopae would be present and ready to settle during spring tides at all times in the breeding season.

Megalopae were expected to reach the upper estuary during spring tides, but little movement occurred at such times. The observed bimodal distribution of the abundance of megalopae during the semilunar cycle may reflect differential rates of larval development or transport among the three species of *Uca*, or temporal variation in hydrographic features that affected the transport of all species equally. Megalopae may have been rare during the syzygies because the tide ebbs during most of the night around the time of the full and new moons in the North Inlet estuary and because megalopae entered the water column primarily during nocturnal flood tides. Any hypothesized selective advantage to megalopae that move up the estuary during spring tides must be less than the advantages to megalopae that move only during the night.

During each summer tide cycle there is a net export of approximately 15% of the brachyuran crab larvae (99% stage I) that are entrained in the water that is tidally pumped across the boundaries of the North Inlet estuary (Christy and Stancyk, 1982). Crab larvae that develop in the ocean may enter the estuary by being transported landward by currents near the bottom (Scheltema, 1975; Sulkin *et al.*, 1980). This study suggests that once *Uca* megalopae occur in tidal creeks, they selectively ride flood tides at night, perhaps moving in a saltatorial fashion to substrates in the upper estuary. *Uca* megalopae were common on intertidal substrates during diurnal low tides following nights of peak abundance, but they were rare when few were caught the previous night. Megalopae that moved past the sampling site at night probably were seeking substrates on which to settle.

The results of this study do not support the hypothesis that cycles of larval release by *Uca* spp. result in maximum rates of transport of megalopae by spring tide currents to substrates in the upper estuary. Rather, the timing of larval release appears to result in rapid seaward transport of newly hatched zoeae on nocturnal ebb tides.

At North Inlet, the amplitude of nocturnal ebb tides is correlated with the semilunar cycle. High tides occur just after sunset during the full and new moons and the subsequent ebb tides are greater in amplitude than those that occur at other times in the semilunar cycle (Fig. 5). Since *U. pugilator, U. pugnax*, and *U. minax* release larvae only at night near the time of high tide, and since peak hatching occurred near the time of the full and new moons, these crabs were releasing larvae at the time in the semilunar cycle when stage I zoeae would be transported most rapidly at night toward the ocean. It is impossible to judge whether larval release occurs in response to factors that vary with the phases of the moon or in response to factors that vary with the amplitude of nocturnal ebb tides because these two cycles coincide at North Inlet. To distinguish between these alternatives, one needs to know when larval release occurs at a site where nocturnal ebb tides of greatest amplitude occur sometime other than during the syzygies.

Reproductive cycles have been described for 17 species of *Uca* (von Hagen, 1962, 1970; Feest, 1969; Zucker, 1973, 1978; Christy, 1978; Wheeler, 1978; DeCoursey, 1981) at sites ranging from the east coast of India to the mid-Atlantic coast of the United States. With one exception, both larval release and the greatest amplitude nocturnal ebb tides during the semilunar cycle occur within about three days of the full and new moons. However, in Charlotte Harbor on the west coast of Florida, both larval release by *U. pugilator* and nocturnal ebb tides of maximum amplitude usually occur during the quarter moons (Christy, 1978; NOAA tide tables). This exception, which provides the only data of use to distinguish between the above alternatives, suggests larval release is timed to occur during large amplitude ebb tides at night, not during a particular phase of the moon.

Semilunar cycles of larval release have been described for 5 species of grapsid crabs (Warner, 1967; Saigusa and Hadaka, 1978; Seiple, 1979; Dollard, 1980), 2 gecarcinids (Gifford, 1962; Henning, 1975; Klaasen, 1975), a xanthid and a pinnotherid (Christy and Stancyk, 1982). All these estuarine crabs release larvae on large amplitude nocturnal ebb tides near the time of the full and new moons. It is unlikely that convergence in such a fundamental feature of the reproductive ecology of these terrestrial, semiterrestrial, and benthic crabs is a fortuitous result of similar adaptive responses to selective factors that operate differently in the diverse habitats of the adults (*e.g.*, Zucker, 1978; DeCoursey, 1979; Bergin, 1981). Convergence among these species more likely reflects a shared adaptive response to mortality factors experienced in common by their meroplanktonic larvae (but see Saigusa, 1981). This study suggests such selective factors must cause higher larval mortality during the day and in the upper estuary than during the night and in the lower estuary or offshore. The following mortality factors may meet these criteria.

On the Atlantic and Gulf Coasts of the United States, larval, postlarval, and juvenile stages of many marine and estuarine spawned fish use the upper reaches of tidal creeks as "nurseries," moving seaward as they grow and mature (*e.g.*, Chao and Musick, 1977; Bozeman and Dean, 1979; Shenker and Dean, 1979; Weinstein, 1979). Planktivorous species such as menhaden (*Brevoortia tyrannus*), silversides

(*Menidia menidia*) and the bay anchovie (*Anchoa mitchilli*) are abundant in the upper estuary during the entire crab breeding season. Other common species such as spot (*Leiostomus xanthurus*) and pinfish (*Lagodon rhomboides*) are planktivorous only when small, in the spring, when crabs begin to breed (Thayer *et al.*, 1974; Kjelson *et al.*, 1975; Chao and Musick, 1977). In general, such species feed on planktonic crustaceans only during the day (*e.g.*, Kjelson *et al.*, 1975; Robertson and Howard, 1978). Predation in the upper estuary by diurnally feeding planktivores may produce powerful selection on when estuarine crabs release larvae. Zoeae that are rapidly transported seaward following release near the peaks of large amplitude nocturnal tides may better escape such predation than those released at other times (see also Bergin, 1978). If true, one would expect crab larvae that complete development in the upper estuary to possess traits that reduce predation.

*Rhithropanopeus harrisi* is found in the extreme upper-reaches of tidal creeks and along rivers at the headwaters of estuaries from New Brunswick to Brazil (Williams, 1965). This xanthid crab releases larvae continuously, its zoeae display patterns of vertical migration that minimize seaward transport, and it completes larval development in the estuary (Williams, 1971; Cronin, 1979). *R. harrisi* zoeae are striking among the xanthid larvae that are common in estuaries on the western Atlantic coast because they possess extremely long rostral and antennal spines (Chamberlain, 1962; Kurata, 1970). Recent experiments demonstrate that these spines, together with the dorsal spine, deter ingestion by small planktivorous fish because they make zoeae too large (approximately 2 mm) to enter their buccal cavities (Morgan, 1981).

Dollard (1980) suggested that larvae released on nocturnal high tides might escape lethal high temperatures. Maximum temperatures are likely to occur during late afternoon low tides in shallow tidal creeks in the upper estuary. At North Inlet in July and August such temperatures commonly exceed 40°C (Dollard, 1980), while maximum temperatures in deeper channels in the middle and upper estuary remain around 30 to 32°C (Bergin, 1978).

Survival rates at high temperatures of first stage *Uca* sp. zoeae depend on salinity (Vernberg and Vernberg, 1975). Fifty percent of stage I *U. pugilator* zoeae die within 1 h, while fifty percent of *U. pugnax* zoeae die within 5 h at 40°C and 20‰. At 35‰ and 40°C, fifty percent mortality of *U. pugilator* and *U. pugnax* zoeae occurs at 12.5 and 6.5 h, respectively. At 38°C zoeae of both species survive over 2.5 d at 20‰ and about 3.5 d at 35‰. Comparable data for the other crabs discussed above are not available.

During summer low tides, larval mortality will be highest in hot, low salinity water in pools and shallow creeks in the upper marsh. Zoeae that are released at high tide just after dark would be transported seaward and might experience relatively high salinities and cool morning temperatures during their first low tide. By migrating vertically with respect to flood- and ebb-directed currents, zoeae might be further displaced seaward into cool high-salinity water during subsequent tidal cycles. Larvae that are released at the peak of large amplitude tides that occur after sunset may best escape lethal combinations of salinity and temperature in both space and time (see also Saigusa, 1981).

I emphasize that future hypotheses about the adaptive significance of the timing of larval release in *Uca* spp. and many other estuarine brachyurans must explain why these crabs release larvae at high tide at night when the amplitude of the nocturnal ebb tides are at a semilunar maximum and zoeae are most rapidly transported away from the upper-reaches of the estuary.

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# ECHINODERM CALCITE: A MECHANICAL ANALYSIS FROM LARVAL SPICULES

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## ABSTRACT

The flexural stiffness ($EI$) was measured for simple and fenestrated spicules in echinoid larvae. A Young's modulus ($E$) of $36.3 \times 10^9$ N/m$^2$ was estimated for these calcitic spicules by $EI/I$ where $I$ was calculated independently from measurements made by SEM. The flexural stiffness of fenestrated spicules is approximately three times greater than that of simple spicules. This increased flexural stiffness is due to structural and not material differences between the spicules. At the material level, this calcitic tissue behaves like a composite which will reduce stiffness but increase strength compared to inorganic calcite. At the structural level its porous nature increases its stiffness and buckling strength over that of a solid structure of similar weight. These characteristics should also increase the tensile strength of this skeletal component and increase its usefulness as a strong, stiff element in most echinoderm skeletons.

## INTRODUCTION

This paper discusses the mechanical properties of echinoderm calcitic tissue (hereafter echinoderm calcite) at the material, structural, and skeletal levels. I present results of bending studies on simple and fenestrated spicules of echinoid larvae. These spicules have the same composition and manner of formation as adult calcitic structures (*e.g.*, Okazaki and Inoué, 1976; Loeper and Pearse, 1981). The simple shape of larval spicules facilitates the measurement of the mechanical properties of echinoderm calcite. The results provide evidence that echinoderm calcite is a composite material and exemplify the consequences of arranging this material into porous structures. In the discussion I generalize the findings of this study to adult structures and propose new ideas which may help explain the unusual structure of echinoderm calcite. This treatment expands the known mechanical role of echinoderm calcite beyond withstanding compressive loads.

## MATERIALS AND METHODS

Echinoplutei with simple and fenestrated skeletons were reared in culture or obtained from the plankton near Friday Harbor, Washington. Culture methods are adapted from Hinegardner (1967) and Strathmann (1971). Spicules from larvae of *Stronglyocentrotus droebachiensis* O. F. Muller, *Strongylocentrotus franciscanus* A. Agassiz, and *Dendraster excentricus* Eschscholtz were isolated with 5% sodium hypochlorite (Chlorox Bleach) and washed three times with distilled water. Adult calcitic structures from the holothurian, *Psolus chitonoides* H. L. Clark were isolated by a similar method.

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Definitions of mechanical terms are as follows:

$\sigma$: Stress—force/cross-sectional area, where the force acts over that area. Units: $N/m^2$.

$\epsilon$: Strain—change in length/original length, where change in length is produced by a stress. Units: dimensionless or m/m.

$E$: Young's modulus—the stiffness of a material, $\sigma/\epsilon$. The stress in a material is divided by the strain produced under stress. Units: $N/m^2$.

$I$: Second moment of area of a cross-section is a description of the geometric distribution of material around a neutral axis of bending. $I = \int y^2\, dA$ where dA is area of material at distance y from the neutral axis (see Wainwright *et al.*, 1976). For a circular cross-section $I = 1/4\, \pi r^4$ where r = radius. Units: $m^4$.

$EI$: Flexural stiffness of a structure—the product of Young's modulus and the second moment of area which describes the ability of a structure to resist bending. Units: $Nm^2$.

*EI* may be used to compare structures which vary in material or shape, and is especially useful when shapes are complex. If either *E* or *I* and flexural stiffness are known, then the other variable may be determined. In this study *EI* is determined for spicules from small scale deflections of two cantilevers: a glass microneedle whose flexural stiffness was determined separately and an experimental spicule. Made from a fiber of fiberglas, the microneedle was 10 $\mu$m in diameter and 1 mm long and was fixed to the end of a 3 mm diameter glass rod. The flexural stiffness of the needle was determined by calculating *I* from dimensions and *E* from equations of bending for a simply supported beam (Gordon, 1978). Known weights (0.2 and 0.5 mg) were hung on the needle and vertical deflection, measured in $\mu$m, was photographed with a horizontally oriented photomicroscope. *E* for the needle is 60 $GN/m^2$.

For measurement of spicule stiffness, a cantilevered spicule was fixed over the edge of a microscope slide with Eastman 910 cement. The needle and spicule were aligned horizontally in the focal plane under a photomicroscope, and the stage was moved so that the needle bent the spicule. Multiple exposure photos were taken because they increase the accuracy in measuring deflection of the needle (Fig. 1). The photographs were analyzed by superimposing the undeflected spicule over the deflected spicule, and then measuring the length of both cantilevers to point of contact and the distance from this point to the identical point on each undeflected cantilever (see Fig. 1). The force exerted on the deflected spicule is equal to that exerted on the deflected needle, so flexural stiffness was determined by solving cantilever bending equations (Gordon, 1978) as follows.

$$F_n = 3(E_n I_n Y_{nmax})/L_n^3 = F_s = 3(E_s I_s Y_{smax})/L_s^3$$

where

$F_n$ = force exerted by the needle of the spicule (Units: N)
$F_s$ = force exerted by the spicule on the needle (N)
$E_n$ = modulus of the glass ($N/m^2$)
$E_s$ = modulus of the spicule ($N/m^2$)
$I_n$ = second moment of area of the needle ($m^4$)
$I_s$ = second moment of area of the spicule ($m^4$)
$Y_{nmax}$ = deflection of the needle where it contacts the spicule (m)
$Y_{smax}$ = deflection of the spicule where it contacts the needle (m)
$L_n$ = length of the needle to point of contact with spicule (m)
$L_s$ = length of the spicule to point of contact with needle (m)

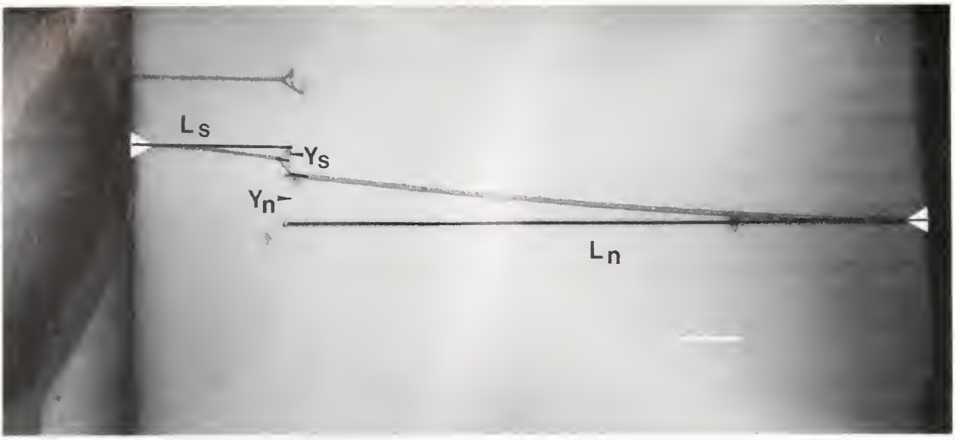


FIGURE 1. Stiffness measuring technique, a double exposure. The spicule was moved to contact and bend the stationary needle, and the first exposure was taken. Then the spicule was moved free of the needle, and a second exposure was taken. The image of the undeflected spicule was then drawn onto the photo. Dimensions were taken from the photographs. Black lines show the length of the cantilever needle, $L_n$, and spicule, $L_s$. $Y_n$ and $Y_s$ are the distances of deflection of the needle and spicule respectively. See text for further explanation. Scale (white line): 0.1 mm.

This equation can be rewritten in the following way:

$$E_sI_s = (Y_n/Y_s)\cdot(L_s/L_n)^3\cdot(E_nI_n)$$

Only bends where $Y_n/L_n$ and $Y_s/L_s \lesssim 10\%$ were used because this equation is not accurate for larger deflections where shear in the material becomes increasingly important (Faupel, 1964). No attempt was made to measure breaking strength.

For simple spicules which are circular in cross-section, $I = 1/4\ \pi r^4$ with r = radius of the spicule. For the fenestrated spicules, $I = 3/4\ \pi r^4 + 3/2\ \pi r^2 d^2$, with r = radius of the element rods and d = radius of the spicule. The amount (volume) of skeletal material in larval spicules was also estimated with dimensions taken from SEM photos. I treat the fenestrated spicules as three parallel circular cylinders joined by cross-ties.

Other mechanical calculations will be introduced as needed in the discussion. They are taken from Wainwright *et al.* (1976) and Faupel (1964), and may be found in most general mechanical engineering texts.

## RESULTS

The spicules of echinoplutei reared during this study are 500 to 800 $\mu$m long. Simple spicules range in diameter from 2 to 4 $\mu$m. Each of the three elements of fenestrated spicules are 1.5 to 2.5 $\mu$m in diameter and the whole spicule is 5 to 10 $\mu$m in diameter (Fig. 2). Fenestrated spicules contain about twice as much material as simple ones of similar length. In a fenestrated spicule the material is located farther from the bending axis, so these spicules should be stiffer than simple spicules. An empirical measure of stiffness rather than calculation of $I$ is used for three reasons. For fenestrated spicules the tapering width and irregular spacing of cross-ties make accurate calculation of $I$ difficult. These spicules are stiffest at the base where the spicule enters the body region of the larva. There is also a slight twist in the member elements of the fenestrated spicule of *Dendraster* (Fig. 2f, g). This 60° rotation is in the same direction for all four of the fenestrated spicules in a larva.

Fenestrated spicules are about three times stiffer than simple spicules (Fig. 3; Mann-Whitney U test, $P < 0.001$). The mean stiffness measured for fenestrated is $14.1 \times 10^{-13} \pm 2.2 \times 10^{-13}$ s.e. and mean stiffness for simple spicules is $3.8 \times 10^{-13} \pm 0.6 \times 10^{-13}$ s.e. $Nm^2$. I report only two data points for the simple spicules of *Dendraster*, but these fall in the same range for simple spicules of *S. franciscanus*. The large variation in $EI$ of the fenestrated spicules (Fig. 3) is probably due to a large variation of $I$.

The calculated Young's modulus of calcite is $36.3\ GN/m^2 \pm 2.9\ GN/m^2$ s.e. ($n = 4$) in the simple spicules which were straight and had a constant diameter. The $E$ calculated for one fenestrated spicule is $48.9\ GN/m^2$. No other values of $E$ for fenestrated spicules were determined because of the difficulty of accurately calculating $I$.

Figure 4 shows some of the calcitic structures found in *Psolus chitonoides*. These structures are typical of those found throughout adult echinoderms.

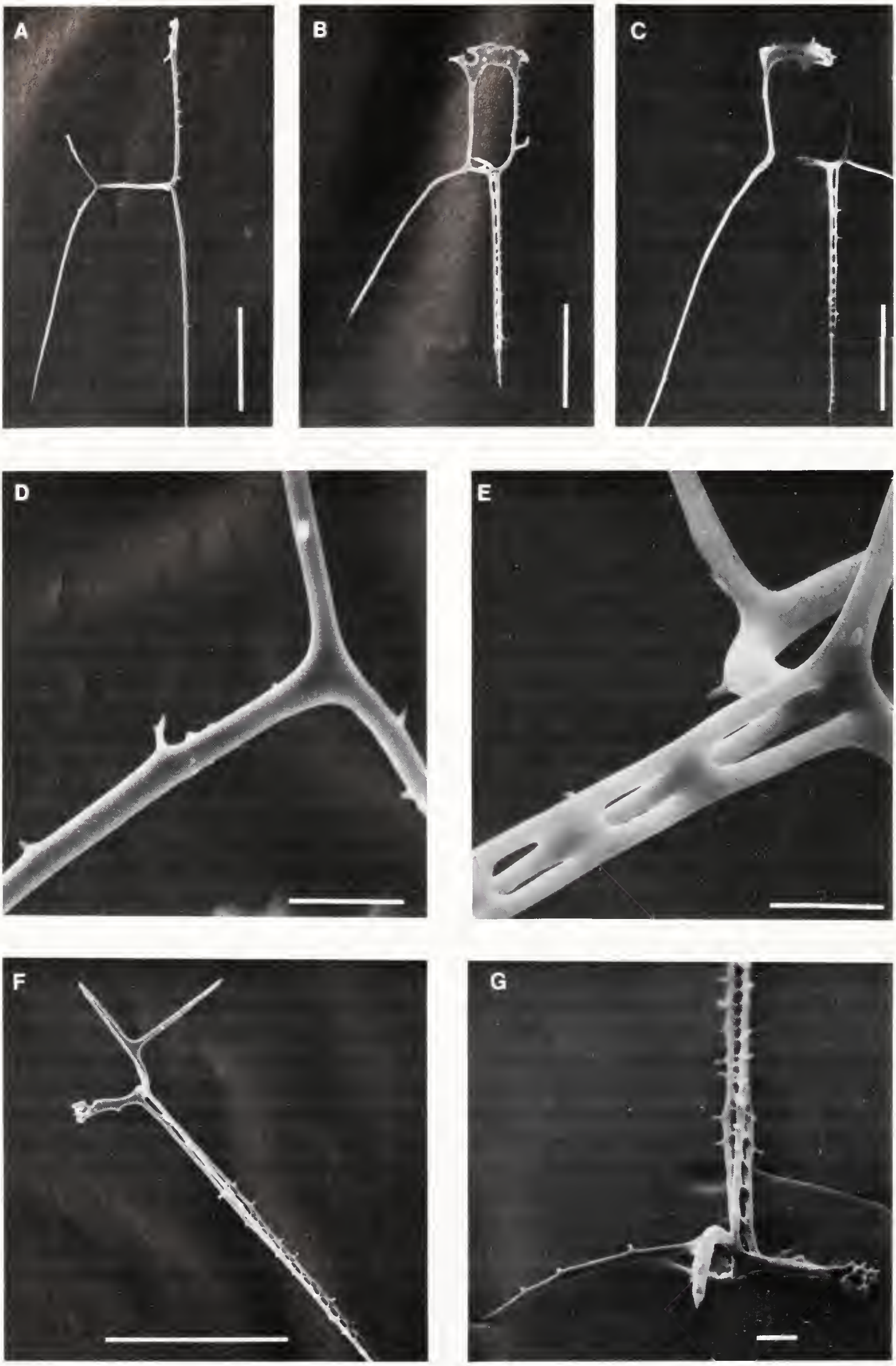
## DISCUSSION

### *The material*

In this study Young's modulus ($E$) of echinoderm calcite is determined to be $36.6\ GN/m^2$ (s.e. = 2.9, n = 4). This value is lower than all previous reports except one. Burkhardt and Märkel (1980) give values for $E$ in diadematid spines as 69.4 and $52.1\ GN/m^2$ for dry and wet spines, respectively. Currey (pg. 167 in Wainwright *et al.*, 1967) gives values of 74 and $9.7\ GN/m^2$ in spines and plates of echinoids, respectively. Differences may be due to methodological difficulties of measuring $E$ and $I$ in previous studies. All of these studies including the present one calculate $E$ from $EI/I$ where $I$ is estimated from cross-sections through the structure and is exclusive of voids in the material. Determination of $I$ can be difficult especially for structures which have a complex distribution of material around a bending axis as in echinoderm stereom, the adult skeletal plate structure. It is possible that $E$ values previously reported differ from what is found here because the $I$ was inaccurately calculated. When $I$ is calculated from dimensions on a photograph, the $E$ is greater for a fenestrated spicule than that for a simple spicule. This is due to an underestimate of $I$, probably due to difficulties of evaluating $I$ at cross-ties. It is not likely that fenestrated spicules are made from a different calcitic material. The simple and fenestrated spicules in *Dendraster* grow out of the same triradiate spicule, and simple spicules have approximately the same $E$ and $I$ as simple spicules from *S. franciscanus*.

As a material, echinoderm calcite should no longer be considered similar to inorganic calcite. The single crystal construction suggested by optical behavior (*e.g.*, Donnay and Pawson, 1969) is more apparent than real. Several authors (Travis, 1970; Pearse and Pearse, 1975; Okazaki and Inoué, 1976; Urakami *et al.*, 1980; O'Neill, 1981) provide evidence for an oriented microcrystalline construction. In addition, all of the reported values for Young's modulus are two to four times lower than that expected for inorganic calcite ($137\ GN/m^2$, Bhimasenachar, 1945). These two differences suggest that there is an organic matrix in echinoderm calcite.

The mechanical properties of a crystalline material may vary with the orientation of the crystal. The modulus of inorganic calcite is $137\ GN/m^2$ in the direction of the C-axis but is as low as $34.2\ GN/m^2$ in the other directions (Bhimasenachar, 1945). Okazaki and Inoué (1976) confirmed that the C-axis in most larval spicules is in the long axis of the spicule. Raup (1966) reports the same for the orientation in spines, but reports that the C-axis may be perpendicular or tangential in echinoid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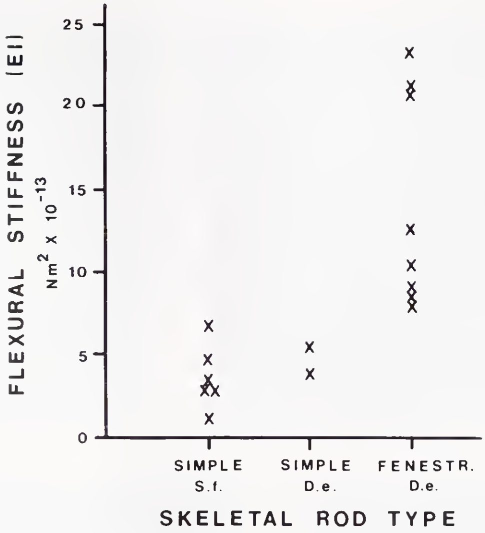

FIGURE 3. Flexural stiffness (Newtons × meter$^2$ × $10^{-13}$) of simple and fenestrated spicules. Mean *EI* of simple spicules: $3.8 \times 10^{-13}$ N/m$^2$, s.e. 0.6. Mean *EI* of fenestrated spicules: $14.1 \times 10^{-13}$, s.e. 2.2. The stiffnesses of the two spicule types are significantly different, Mann-Whitney U test, $P < 0.001$. S.f., *Stronglyocentrotus franciscanus*; D.e., *Dendraster excentricus*.

plates. The orientation of the C-axis along the long axis of these structures means that they are stiffer than they would be if the C-axis were in any other orientation. With the possible exception of the value for the plate, the lower stiffness of echinoderm calcite cannot be attributed to varying C-axis orientation.

Magnesium replaces up to 16% of the calcium in echinoderm calcite, but the reasons for the variation in magnesium content remain obscure (Chave, 1954; Weber, 1969). It is not clear how magnesium content will affect the modulus. Increasing magnesium content increases the hardness of calcite (Wainwright *et al.*, 1976) and therefore will probably increase stiffness.

A porous microstructure would explain the reduced stiffness. Okazaki and Inoué (1976) showed a high magnification SEM photo suggesting a porous surface on carefully isolated spicules. Observation under high magnification of the spicules isolated by my own techniques never revealed that apparent texture. An empirical formula (by Mackenzie, pg. 157 in Wainwright *et al.*, 1976) for change in modulus in a porous ceramic predicts that a 50% volume of pores is necessary to give a 75% reduction in modulus, equivalent to the *E* reported here. Therefore, a porous microstructure probably cannot account for the reduced stiffness of echinoderm calcite.

An organic matrix and composite construction would also reduce the stiffness of the calcitic tissue. Though the collagen connecting the calcitic plates may contaminate some samples (Klein and Currey, 1970; Travis, 1970), there is growing evidence for an organic matrix in echinoderm calcite (Klein and Currey, 1970;

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FIGURE 2. Simple and fenestrated calcareous spicules isolated from echinoplutei. (A) Simple "half skeleton" of two week old *S. franciscanus*. (B, C) Early and later stages of the fenestrated "half skeleton" of *D. excentricus*. In (C) the skeleton is modified and allows articulation of the fenestrated post-oral rod. A, B, C, scale 100 μm. (D, E) Higher magnification of the simple and fenestrated spicules. Note the smooth surface of the calcite. D, E scale: 10 μm. (F, G) Fenestrated spicules of *D. excentricus*. (F) Note the taper and irregular spacing of cross-ties in fenestration. Scale: 100 μm. (G) Same spicule as (F), note the twist in the parallel elements of the fenestrated part. Scale: 10 μm.

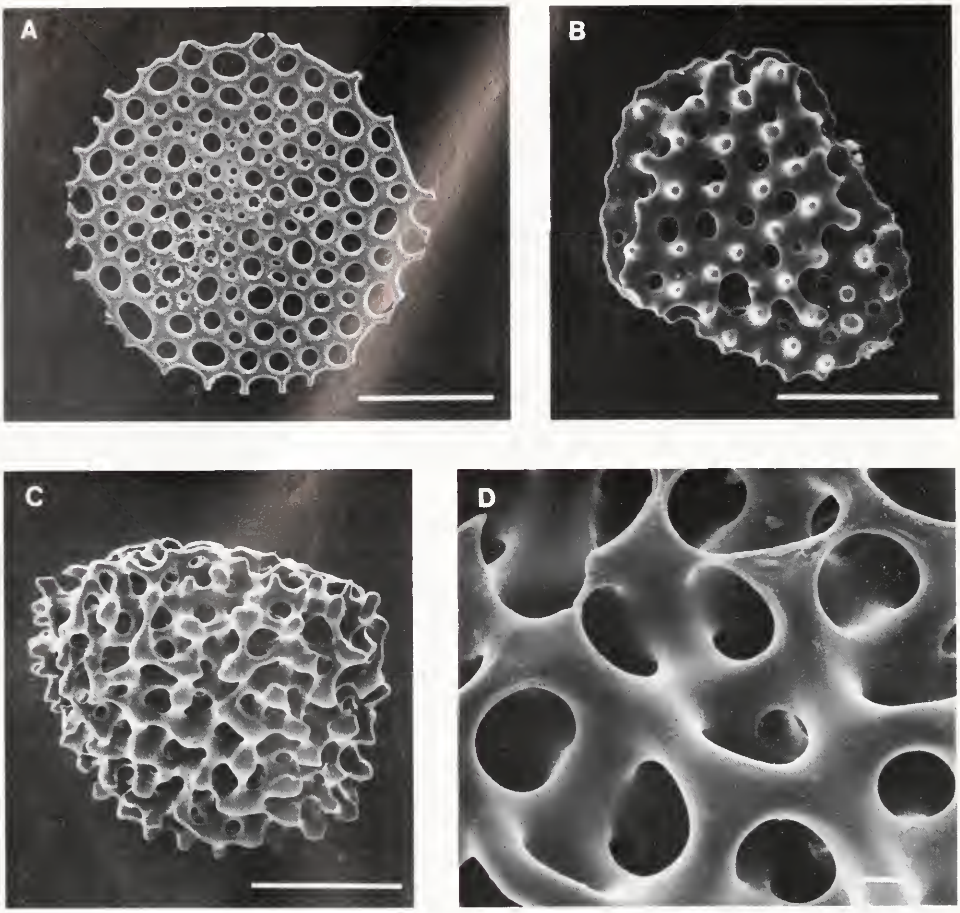

FIGURE 4. Plates and stereom of the holothurian *Psolus chitonoides*. (A) Flat plate. Scale: 100 μm. (B) A plate that is becoming a laminated structure with the addition of a new layer. Scale: 100 μm. (C) Labyrinthic stereom structure in the form of a block. Scale: 100 μm. (D) Higher magnification of a stereom surface. Scale: 10 μm.

Travis, 1970; Pucci-Minafra *et al.*, 1972; Pearse and Pearse, 1975; Okazaki and Inoué, 1976). Okazaki and Inoué (1976) give an organic content for the larval spicules of about 1% by weight. Klein and Currey (1970) give a value of 0.3% protein by weight (about 1% by volume) which is close to the 0.36% for protein in larval spicules given by Okazaki and Inoué (1976).

Evidence for a highly oriented microcrystalline structure in echinoderm calcite is also increasing. Polarized light and X-ray diffraction studies (Raup, 1966; Donnay and Pawson, 1969; Nissen, 1969) suggest that echinoderm calcite is a single crystal, but these studies cannot distinguish between a single crystal and a highly ordered microcrystalline construction where all the microcrystals have the same C-axis orientation. Fracture studies do not show cleavage planes expected of inorganic crystalline calcite (Raup, 1966; Nichols and Currey, 1968; Nissen, 1969; Okazaki and Inoué, 1976), and several authors show fractures (Pearse and Pearse, 1975; O'Neill, 1981) or etching (Okazaki and Inoué, 1976) which suggest concentric laminated ordering of microcrystals. Recent studies by O'Neill suggest that when echinoderm calcite is stressed in tension the microcrystals creep, or move with respect to one another. Currey (1965) loaded echinoid spines in bending and found no creep after 26 h. But, as he states, the spines were from dried specimens, which may have prevented creep from occurring.

If, in fact, this calcite tissue is a highly ordered "inorganic polycrystalline aggregate" (Travis, 1970) bound in a very small amount of organic matrix (1 to 2% by volume), then mechanically its behavior can be treated as a composite material. In this treatment the microcrystals are analogous to short fibers and the organic material is the matrix which binds them. The modulus, $E_c$, of the composite, modeled as a series of layers of fibers and matrix can be predicted as follows:

$$1/E_c = V_f/E_f + V_m/E_m$$

where

$V_f$ = volume fraction of fibers = 99%
$E_f$ = modulus of fibers = 137 GN/m$^2$
$V_m$ = volume fraction of matrix = 1%
$E_m$ = modulus of matrix = 0.6 GN/m$^2$

and $V_f + V_m = 1$

(Currey, pg. 145 in Wainwright *et al.*, 1976. The value of the modulus for the matrix is that of human tendon and is meant to be an approximation to the collagen-like component of matrix.)

By this formula $E_c$ is evaluated to be 41.9 GN/m$^2$ which is one standard deviation higher than the value of 36.3 GN/m$^2$ determined in this study. Therefore, it may be reasonable to treat the material, echinoderm calcite, as a special kind of composite with a high fiber content. Although this formula is used to model composites whose components are arranged in series (Reuss model), there is no evidence that components in echinoderm calcite are physically arranged in this way. The formula merely predicts this composite's behavior. Echinoderm calcite has been called a composite by Weber *et al.* (1969), but the present work is the first to describe its mechanical behavior as a composite material.

### *Biological implications*

Comparison of the mechanical properties of echinoderm calcite with that of inorganic calcite reveals the biological advantages in composite construction. A spicule of composite construction should be effectively stronger than one constructed from a single inorganic crystal. In theory the inorganic calcite should have a higher fracture stress (greater force per unit area at failure), but in practice tiny cracks and surface flaws set the upper limit to fracture stress (Wainwright *et al.*, 1976). A composite construction of many tiny crystallite 'fibers' may reduce the possibility of this common cause of failure in brittle materials if cracks are not propagated through the material when a single or a few fibers break (Wainwright *et al.*, 1976; Gordon, 1978).

Brittle materials are usually weaker in tension than compression. This restricts the usefulness of such a material to sustaining compressive loads. The composite construction should increase the tensile strength over that of inorganic calcite. Fractures caused by rapid loading usually do not show inorganic fracture planes, which would require the lowest work of fracture. In a composite material, in which the modulus of the fibers and the viscosity of the matrix are high, rapid loading should crack through matrix and fiber, but under low and even stress, the matrix would be expected to shear. O'Neill's (1981) pictures of microcrystals in prestressed fracture support this prediction of material behavior.

### *The larval spicules*

Arm rods of echinoderm larvae are the simplest echinoderm skeletons and, therefore, are a good starting place for the analysis of mechanical properties at the structural level. If $E$ is the same for all echinoderm calcite, then comparison of stiffness for different structures can be made through their $I$ values. (Compare $I = 3.9 \times 10^{-23}$ $m^4$ for fenestrated spicules with $I = 1.2 \times 10^{-23}$ $m^4$ for simple spicules.) While the $I$ of fenestrated spicules is approximately three times as much, they contain only twice as much calcite as simple spicules. If the same volume of material that is in a fenestrated spicule were arranged in a simple structure around the bending axis, its $I$ value would be about twice that of the simple spicule. Fenestration gives the spicule an increased stiffness per amount of material. Further, a solid spicule constructed with the same dimensions as a fenestrated spicule would increase the stiffness by an order of magnitude over the simple spicule but would also require about six times the amount of material. The use of less material must be important in a planktonic larva which has to overcome gravity to stay afloat.

Other benefits of fenestration can be appreciated by looking again at the structure (Fig. 2e, f, g). Three parallel rods are the minimum above one which give almost even stiffness around a central bending axis. Two parallel rods will not provide an even distribution of $I$. The left-handed twist of about 60° in the parallel elements reduces stress along any rod when the spicule is bent in a certain direction. This slight twist, in the same direction for all fenestrated spicules in *Dendraster* larvae, probably reflects the construction pattern or orientation of the organic matrix. This pattern cannot be the result of net torque on the arm due to swimming currents, because the mirror image pairs would have opposite coiling twists since the currents are subject to bilateral symmetry.

Fenestration increases stiffness in torsion about a central axis. Here cross-ties increase the $J$ value (second polar moment of area), which is a measure of the geometric distribution of material around a twisting axis and is analogous to the $I$ value. Fenestration also increases the resistance to buckling since it is proportional to flexural stiffness. Functions of the larval skeleton will be discussed in more detail in a later paper.

### *The adult skeleton: porous plates and stereom*

The mechanics of the unusual structure of the adult skeletal plates, called stereom (Fig. 4c,d), have been largely uninvestigated. Nichols and Currey (1968) suggest that the porous structure may strengthen echinoderm calcite since small cracks stop when they run into a hole and also point out that this construction allows access to surfaces for repair. As in the composite construction, the porous structure should reduce the difference between strength in tension and strength in compression. Currey (1975) compared the crushing strength of echinoderm stereom with that of mollusc shell and found it comparable to moderately strong mollusc shell on a unit weight basis. The crushing strength for echinoderm stereom is 50–100 $MN/m^2$ (Wainwright *et al.*, 1976). However, I calculated a stress of 120 $MN/m^2$ at 14% tip deflection in an unbroken spicule during a bending trial (see Wainwright *et al.*, 1976, pg. 248, for formula of tensile stress in bending).

Like fenestrated spicules, the porous calcite structures of adults have the benefits of increased bending and torsional stiffness and buckling strength, when compared on a unit weight basis with solid structures. For structures with the same general shape and composed of the same weight of material, stiffness will increase faster than strength with increasing porosity. This is because stiffness is proportional to *I* which is proportional to $r^4$ and strength is proportional to $I/r$ or $r^3$. *I* will be greater for complex stereom than for solid structures because material is separated in space around the bending axis (larger r). The largest *I* occurs if the material is distributed in an annulus about the central axis. This means that while there are no great differences in the strength to weight ratios of echinoderm stereom and mollusc shell, there are differences in the stiffness or buckling strength to weight ratios, with echinoderm skeleton being greater in both.

Mechanical properties may vary within and between skeletal blocks because of different stereom structure. Smith (1980) demonstrated a range of variation in pore density and pattern in the stereom structure of echinoids and described ten distinct stereom types. Macurda *et al.* (1978) described four of these types for recent crinoids. Figure 4 shows three of these types also found in the holothurian, *Psolus chitonoides*. Reoccurring stereom fabrics suggest the possibility of mechanical differences, but no work has demonstrated this. A more quantitative analysis is needed that will demonstrate how, for a given amount of material, stiffness and strength are influenced by porosity. This analysis may be done by comparing the different stereom structures by calculating *I*'s and cross-sectional areas of material in structures with the different stereom types. Carter and Hayes (1976) showed that different types of bone tissue can be treated similarly in mechanical testing, and that variation in compressive strength in bone of different tissue morphology can be described as a function of its relative density and the compressive strength of compact bone. Similar studies on echinoderm stereom should lead to development of formulae which describe strength or stiffness as a function of density and stereom type.

### *The skeletal system*

The organization of these plates into functional skeletons for organs and organisms is highly varied. Skeletal blocks with different mechanical properties are arranged and interconnected more or less tightly with collagen fibers (Hyman, 1955) and often articulated with muscle. The nature of formation of these optical crystalline blocks inside a syncitium (Okazaki and Inoué, 1976; Loeper and Pearse, 1981) may account for the small degree of variation (mineral content) in the composite material echinoderm calcite. Structural differences may be viewed as the method of varying mechanical properties of calcite materials. Smith (1980) reported that galleried stereom is always associated with long bundles of collagen fibers. Macurda *et al.* (1978) found characteristic spines on the labyrinthic stereom where muscles attach. One of the intensions of Smith's (1980) study was to correlate stereom type with soft tissue type, but perhaps it may be more appropriate to correlate stereom type with mechanical operation.

The porous structure of echinoderm calcite increases its flexural and torsional stiffness, buckling strength, and possibly its tensile strength on a unit weight basis over that of a solid construction. A composite and porous construction may allow wider application of this element in skeletons than just carrying compressive loads. Eylers (1976) describes the distribution of forces in the skeleton of an asteroid during the opening of bivalve prey. The ossicles joined along the aboral surface by collagen and muscle are in tension, and ambulacral ossicles experience bending, torsion, and compression. Tensile forces also occur in the arms of suspension feeding crinoids and ophiuroids, spines of echinoids, imbricate plate systems, and most other examples of echinoderm skeleton. The mechanical behavior of an intact adult skeleton should then be analyzed as an interaction between composite blocks, collagen connective tissues, and muscle. The mechanical diversity of echinoderm skeletal organization may also be attributed to material and structural properties of echinoderm calcite.

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# MECHANISM OF THE EXCITATION-CONTRACTION UNCOUPLING OF FROG SKELETAL MUSCLE BY FORMAMIDE

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## ABSTRACT

The contractility of guinea pig ileum and frog skeletal muscle is inhibited in solutions containing 0.4 to 2.5 *M* formamide (FMD). Contrary to mammalian visceral muscle, this blocking action is not reversed when frog muscles are transferred back to isotonic Ringer's after FMD treatment. Under these conditions the water content of the skeletal muscles is markedly increased and electronmicrographs show a swelling of the transverse tubules. These changes are not observed when frog muscles are transferred to ethylene glycol solutions that are isosmotic with the FMD containing Ringer's solution. In addition, over 50% of the contractility is recovered in these muscles. These observations provide direct evidence of the occurrence of an osmotic shock in frog muscles transferred from FMD solutions to isotonic Ringer's. It is concluded that the resulting alterations in the triad structure and function are responsible for the irreversibility of the FMD uncoupling action in these muscles.

## INTRODUCTION

Formamide (FMD), added to isotonic saline at concentrations between 0.4 and 2.5 *M*, produces an immediate and completely reversible inhibition of the shortening of mammalian visceral muscle (Córdoba *et al.*, 1968) and blocks irreversibly with a slower time course the contractility of frog skeletal muscle (del Castillo and Escalona de Motta, 1978). The skeletal muscle fibers blocked by FMD retain their electrical and chemical excitability properties and are able to respond with fast local twitches to the electrophoretic injection of $Ca^{2+}$ (Escalona de Motta and del Castillo, unpublished observations). In addition, caffeine still induces slow sustained contractures in these muscles (Escalona de Motta *et al.*, 1982). These observations suggest that the effects of FMD on frog skeletal muscle are exerted on the coupling between excitation and contraction. In this sense, FMD may be classified, together with glycerol and ethylene glycol, as an excitation-contraction (E-C) uncoupling agent.

However, the uncoupling action of glycerol and ethylene glycol does not occur until the muscles are suddenly transferred back to isotonic Ringer's, inducing an osmotic shock that disrupts the tubules of the T-system. (Eisenberg and Gage, 1967). With FMD, muscles lose their contractility while still immersed in the hypertonic

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Abbreviations: E-C, excitation-contraction; EG, ethylene glycol; FMD, formamide; SR, sarcoplasmic reticulum.

solution, indicating that an osmotic shock is not essential for the uncoupling action exerted by FMD (del Castillo and Escalona de Motta, 1978). The present work investigates the possible osmotic effects of FMD solutions on frog sartorius muscles, measuring changes in the water content of these muscles and examining the ultrastructure of the muscle fibers under various conditions.

## MATERIALS AND METHODS

*Preparations.* Sartorius muscles of small (2″) frogs (*Rana pipiens*) were dissected, with or without the sciatic nerve attached, and pinned to a layer of Sylgard (Dow Corning) at the bottom of a small Petri dish. Contractility was determined visually by observing the twitches induced by stimulating the muscle directly or via the attached motor nerve using a pair of platinum electrodes. All the applied stimuli were square pulses of 1 ms duration and supramaximal strength. In experiments where the tension developed was measured, the muscles were tied at both ends with silk threads and placed in a vertical bath containing Ringer's solution. The muscle, attached to the chamber by one end, was connected by the other end to a Grass FT 103 isometric transducer connected to a chart recorder.

*Physiological solutions.* The mammalian Krebs-Ringer's solution employed had the following ionic composition (m*M*): $Na^+$, 118; $K^+$, 4.6; $Ca^{2+}$, 2; $Mg^{2+}$, 0.9; $Cl^-$, 117; $HCO_3^-$, 17.6; $SO_4^{2-}$, 0.9; $H_2PO_4^-$, 0.9. A mixture of 98% $0_2$ and 2% $CO_2$ was bubbled continuously through the solution, which had a pH of 7.3 after equilibration. Glucose (5 m*M*) was added to this solution. Direct measurement of osmolarity with a cryosmometer gave values ranging between 295 and 305 mOsm/liter.

The frog Ringer's solution used contained the following ionic concentrations (m*M*): $Na^+$, 117; $K^+$, 2.1; $Ca^{2+}$, 1.87, all as chloride salts. The pH of this solution was adjusted to 7.2 with 5 m*M* TES (N-tris hydroxymethyl methyl-2- aminoethanesulfonic acid) and NaOH. The osmolarity of this solution, determined with an Advanced Instruments osmometer, ranged from 242 to 250 mOsm/liter. FMD or ethylene glycol (EG) was added to these solutions in the concentrations further indicated.

*Measurement of water content in muscle.* The muscles were weighed at regular intervals before and after they were immersed in Ringer's solution to which different amounts of FMD had been added. Extreme care was taken in handling the muscles to ensure reproducibility of the results. After each series, the muscle was dried over a desiccant, until there was no further change in weight. The water content of each muscle was calculated by subtracting the dry weight from the original wet weight.

*Ultrastructural experiments.* Muscles which were in normal Ringer's saline were fixed in a solution containing 2% glutaraldehyde in 0.05 *M* cacodylate buffer (pH 7.4) with 4% sucrose for 24 hours at 4°C, and the solution was changed several times during the first hours.

Other muscles were transferred directly from a 2.0 *M* FMD solution in Ringer's to a fixative like the one above, but also containing 2.0 *M* FMD.

Finally, a third group of muscles was sequentially transferred from 1.0 *M* FMD to isosmotic ethylene glycol and then to the basic fixative solution to which 1.0 *M* of ethylene glycol had been added.

All specimens were treated subsequently with 1% osmium tetroxide in 0.05 *M* cacodylate buffer for 1 hour at 4°C, dehydrated in an ethanol series, and embedded in Araldite. Sections were cut with glass or diamond knives on an LKB Ultratome III, stained with ethanolic uranyl acetate and lead citrate, and examined in a Philips EM 400 and 200.

## RESULTS

*Osmotic effects of high FMD concentrations.* FMD is a highly permeant solute, as shown by the fact that the water content of ileal strips placed in 2 *M* FMD in Krebs-Ringer's solution show no appreciable change in weight after 20 min. In addition, as shown in Figure 1, no significant changes in water content could be detected when these ileal strips were transferred back to normal Krebs-Ringer's.

The movement of FMD across the membrane systems of skeletal muscle is slightly more restricted. Indeed, frog sartorius muscles exhibit a small (15%) decrease in total weight when immersed in 2 *M* FMD-Ringer's, and almost double their water content when transferred back to normal Ringer's (see Figure 2). This last observation suggests that FMD does not leave the skeletal muscle fibers as easily as it goes into them, thus favoring the occurrence of an osmotic shock similar to that brought about in skeletal muscles exposed to hypertonic glycerol (Gage and Eisenberg, 1969).

*Ultrastructure of muscles equilibrated in hypertonic FMD.* To determine whether the block of contraction which occurs while muscles are still immersed in the FMD solution could be correlated with ultrastructural changes caused by hypertonicity, two muscles blocked after 18 min in 2.0 *M* FMD were transferred to a fixing solution that also contained 2.0 *M* FMD. Post-fixation and further processing of these specimens was then done as described in Materials and Methods. Figures 3 and 4 show that the myofibrils and associated sarcoplasmic reticulum (SR), as well as the membranes of the tubular system (T-system), of these muscles are normal in appearance and do not present any obvious morphological features that may be associated with a loss of contractility.

*Structural changes in FMD-blocked muscles transferred to normal Ringer's.* Two muscles blocked after 18 min in 2 *M* FMD-Ringer's were transferred to isotonic Ringer's and washed extensively for 30 min before fixation.

Figure 5 and 6 illustrate the marked changes in ultrastructure observed. There are large numbers of "lacunae" irregularly distributed throughout the fields but

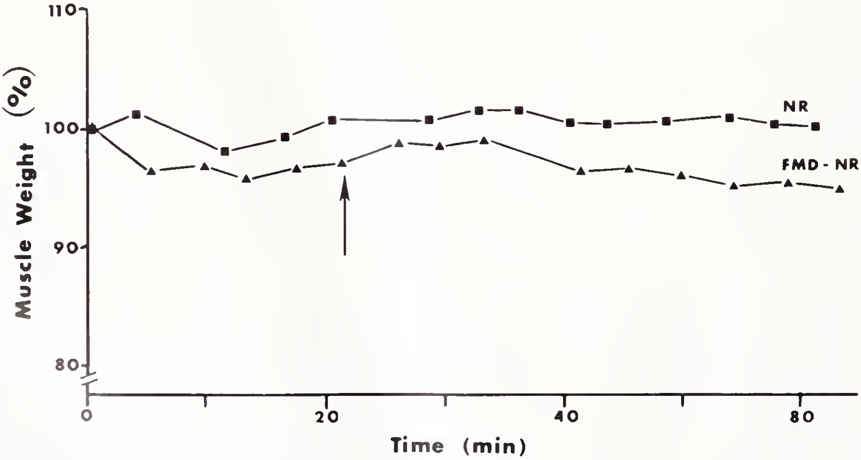

FIGURE 1. The water content of guinea pig ileum strips does not change appreciably upon exposure to 2 *M* FMD and after transfer to normal Ringer's (NR). Arrow indicates the change from FMD or NR. Top curve is the control muscle maintained in NR throughout the experiment. See Materials and Methods for experimental procedure.

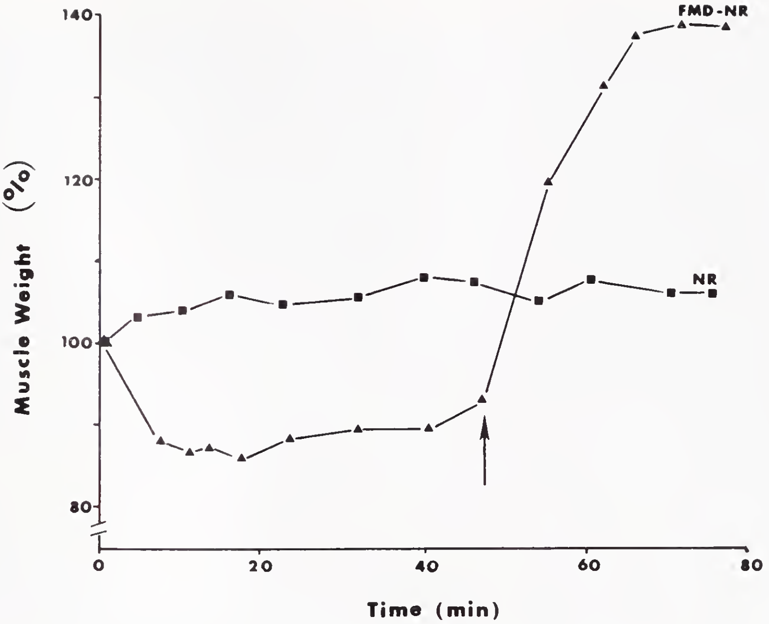

FIGURE 2. Frog sartorius muscles equilibrated in 2 $M$ FMD increase markedly their water content when brought back to normal Ringer's (arrow). Muscle marked NR was maintained in normal Ringer's throughout the experiment. Experimental procedure is explained in Materials and Methods.

always lying at the level of the Z bands of the myofibrils. In these regions, the T-tubules are swollen but maintain their close association with the membrane of the terminal cisternae of the SR.

*Recovery of contractility in FMD-blocked muscles transferred to isosmotic EG.* To test whether the observed osmotic shock was *per se* the cause of the irreversibility of the uncoupling action of FMD on skeletal muscles, we performed experiments avoiding the occurrence of drastic osmotic changes. Pairs of sartorius muscles were exposed to a 1.0 $M$ FMD solution (1,190 mOsm) for a period sufficiently long to produce a complete loss of contractility at this lower FMD concentration. These muscles were then transferred to a 1 $M$ EG solution (1,210 mOsm). Ethylene glycol has been employed in similar concentrations to produce E-C uncoupling of frog muscles by an osmotic shock (Sevcick and Narahashi, 1972), but, by itself and in contrast to what we have shown with FMD, it does not impair muscle contraction (Caputo, 1968).

Both the water content of the muscles and their contractility in response to direct electrical stimulation were determined in these preparations. Figure 7 shows that the water content of these muscles decreases slightly after transfer to the EG solution, indicating that this alcohol does not penetrate freely into the muscle fibers. Figure 8 illustrates the time course of the blockade of a muscle exposed to 1 $M$ FMD and the partial recovery of contractility after 24 min in EG. As emphasized in the discussion, the reduced force of contraction of muscle treated by FMD may be due to the occlusion of a fraction of the T-tubules.

*Structure of muscles fixed after successive exposure to FMD and EG.* Figure 9 shows a survey field of a sartorius muscle fiber exposed sequentially to 1.0 $M$ FMD and isosmotic solution prior to fixation with a glutaraldehyde solution that also contained 1.0 $M$ EG. The appearance of the relaxed myofibrils and the associated

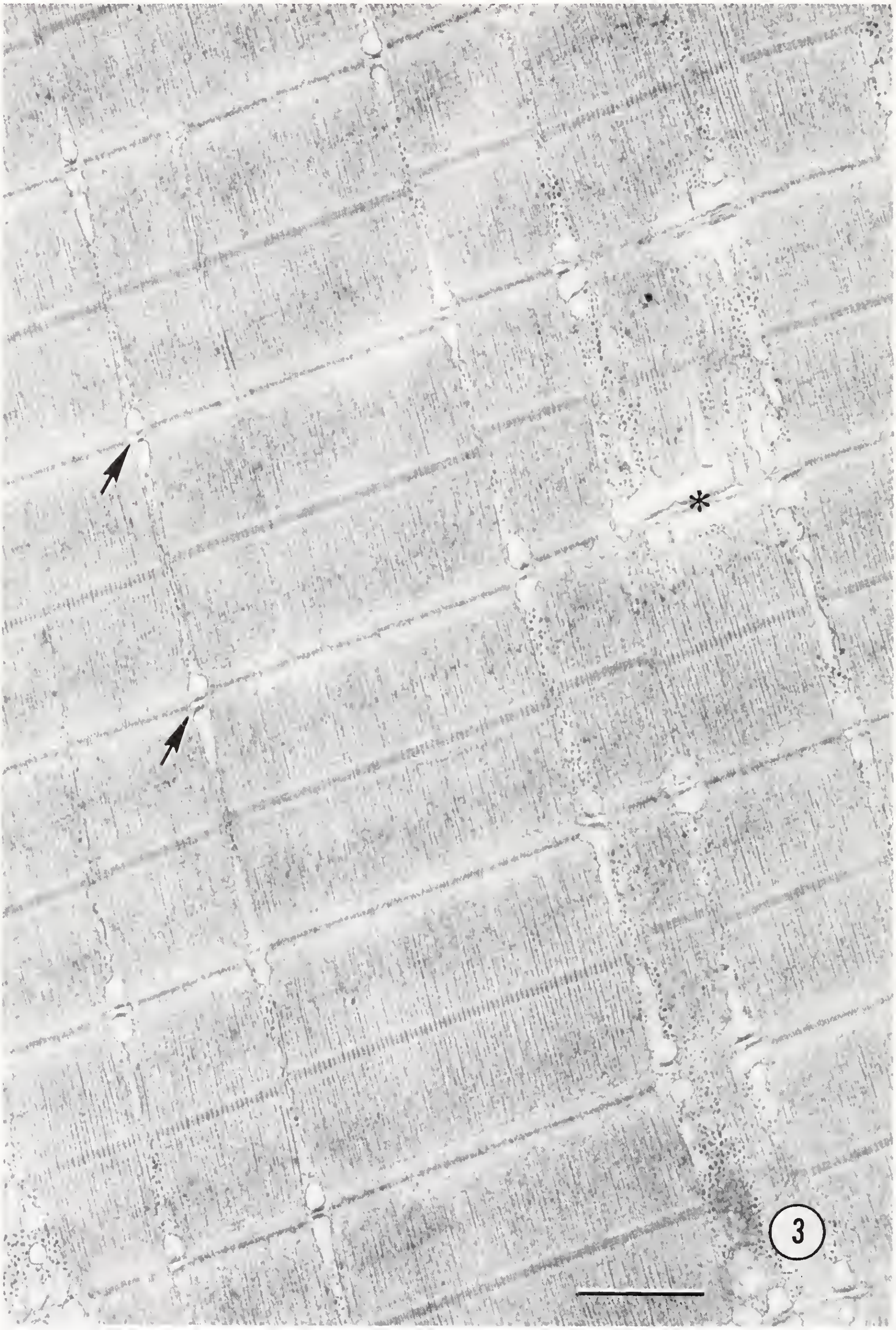

FIGURE 3. Frog sartorius muscle fixed in glutaraldehyde in presence of 2 *M* formamide (see Materials and Methods). The myofibrils (in this instance contracted) and associated SR and T-system membranes are essentially normal in appearance. Arrows indicate triads situated, as is characteristic of this muscle, at the Z band level. Scale bar: 1 $\mu$m.

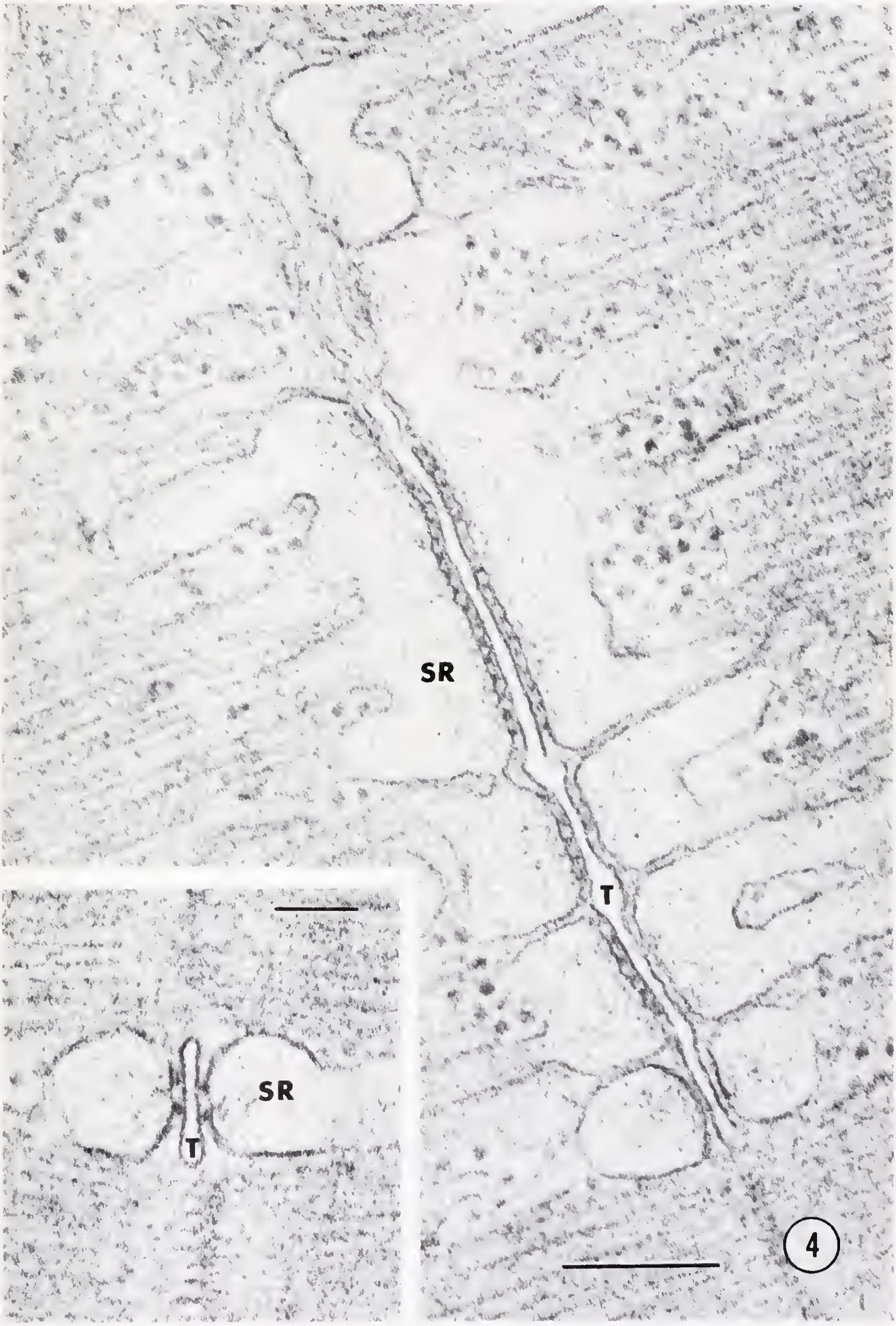

FIGURE 4. Material prepared as in Figure 3, illustrating details of the triad configurations. This field includes an extensive T-tubule profile (T), flanked by terminal cisternae (SR). The width of the triad gap and the spacing of the foot or pillar processes stemming from the SR are as in conventionally fixed material. The components of the triad are further illustrated in the inset, in which the T-tubule is transversely sectioned: note the normal disposition of the foot processes. Scale bars: 0.25 μm; inset, 0.1 μm.

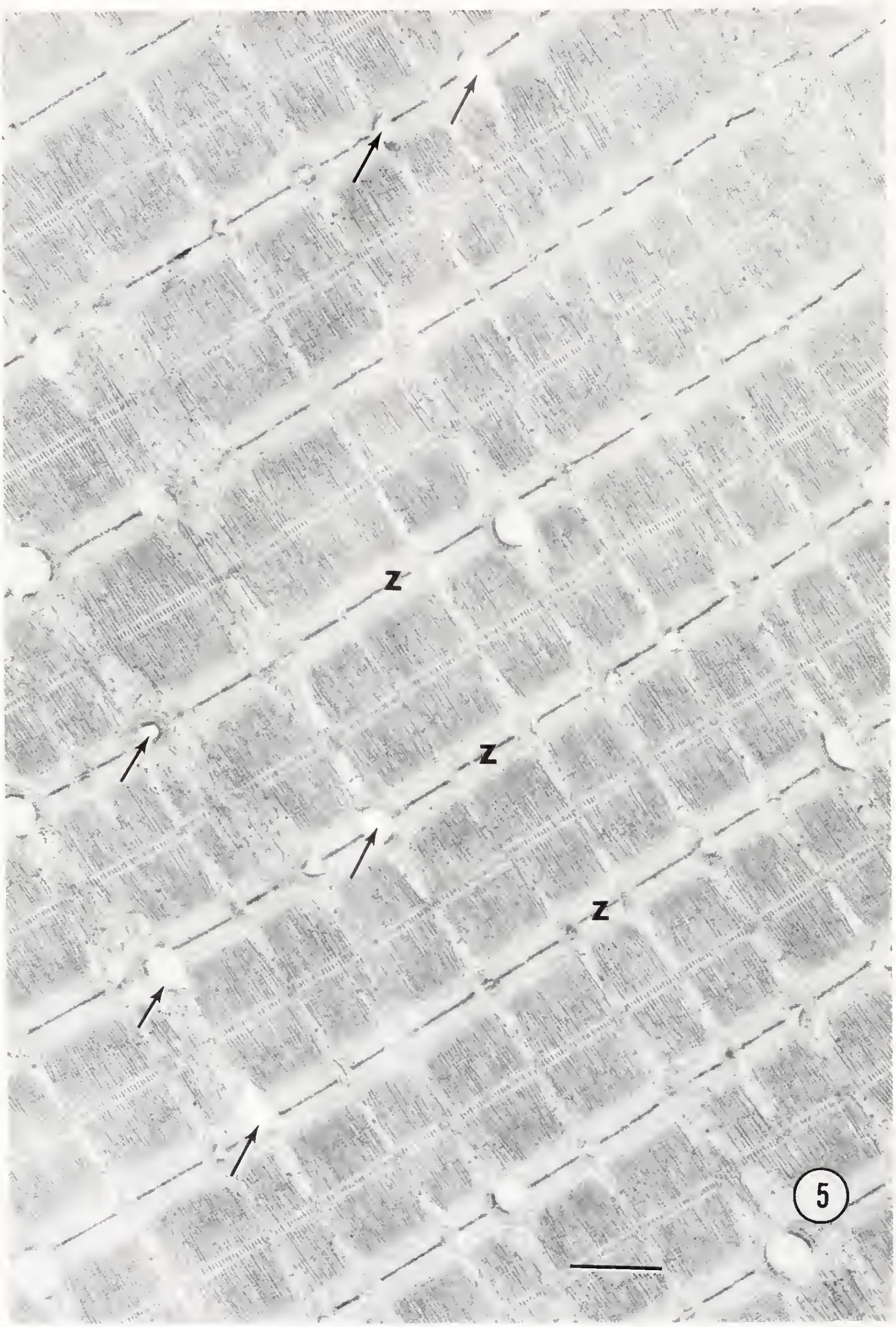

FIGURE. 5. Frog sartorius muscle sequentially soaked in 2 *M* formamide, washed in Ringer's and fixed in glutaraldehyde. (See Materials and Methods for times of treatment). In this survey field, the obvious abnormality is the presence of 'lacunae' (arrows), irregularly scattered through the material, of varying size but invariably lying at the Z band levels. These are further illustrated in Figure 6. Scale bar: 1 $\mu$m.

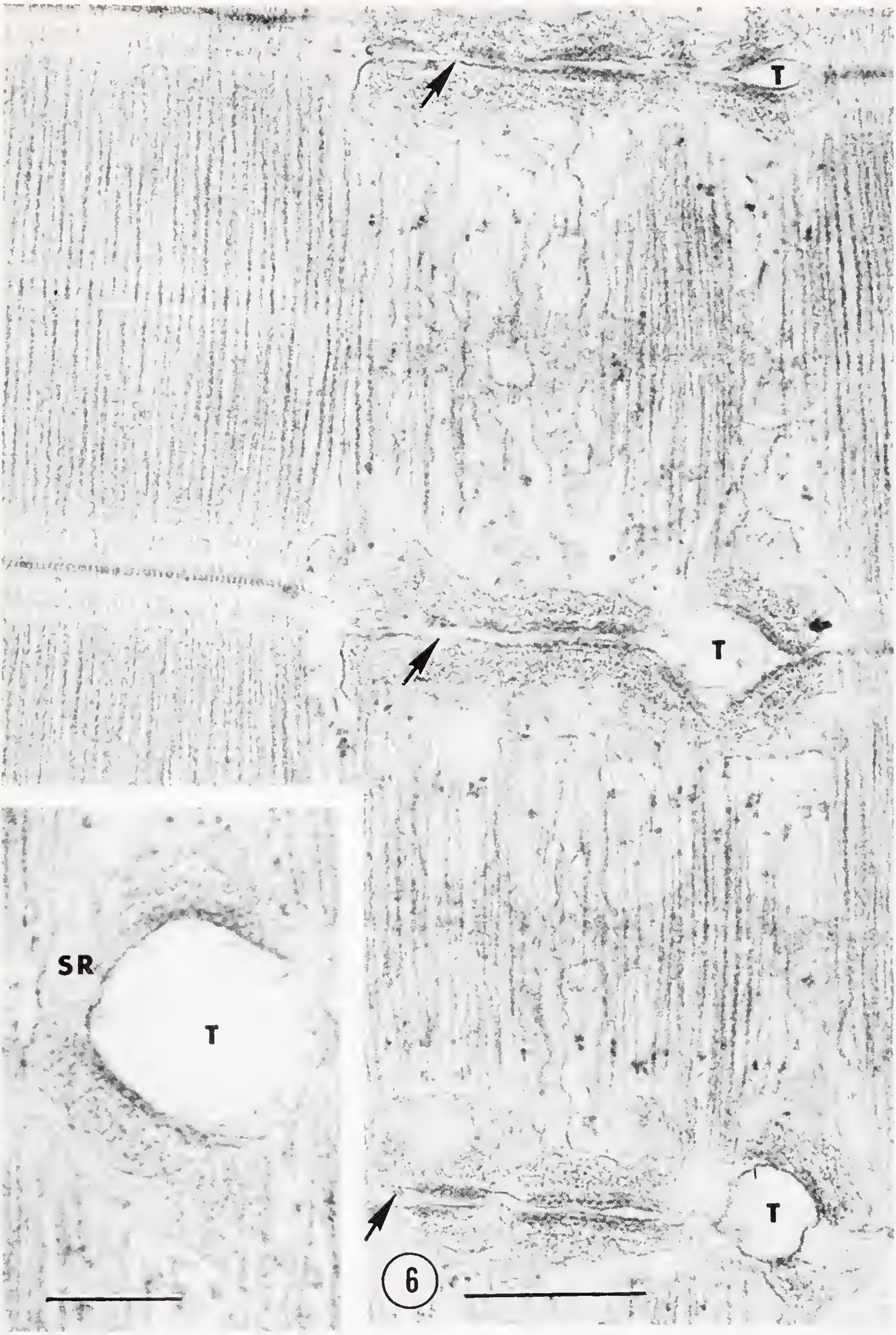

FIGURE 6. Material fixed as in Figure 5, at higher magnification. Portions of three triads are included in tangential section in this field, encircling the underlying myofibril. These are 'normal' in appearance for part of their course (arrows) but on the right of the field (T) the medial T-tubules are swollen to varying degrees, providing the Z-level 'lacunae' seen in Figure 5. As in this instance, the swelling is often irregular along the course of an individual tubule, occurring primarily between or at the periphery of the circumfibrillar triads. However, as shown in the inset, the swelling sometimes affects the triad itself. This micrograph includes a severely abnormal triad: the T-tubule is grossly swollen, but retains its original association with the terminal cisternae (SR). Scale bars: 0.5 $\mu$m; inset, 0.25 $\mu$m.

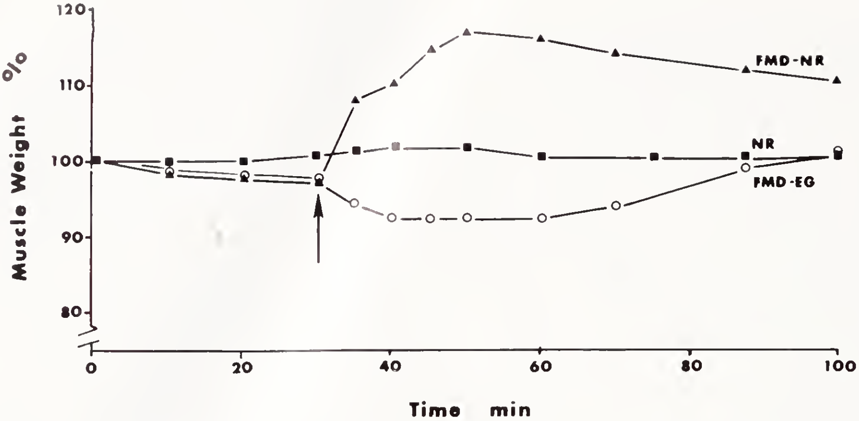

FIGURE 7. Changes in the water content of two frog sartorius muscles equilibrated with 1.0 $M$ FMD and transferred to either normal Ringer's (FMD-NR) or 1.0 $M$ EG (FMD-EG). The curve marked NR was obtained with a muscle maintained in normal Ringer's throughout the experiment. Arrow indicates the moment in which the muscles were changed from the FMD solution. See Materials and Methods for an explanation of the experimental procedure.

triadic junctions is essentially normal. In particular the tubular swellings obvious in Figures 5 and 6 are absent.

## DISCUSSION

The results of the experiments described above demonstrate the occurrence of an osmotic shock in frog sartorius muscles transferred from FMD solutions to normal isotonic Ringer's. The fact that in any given field of the electron micrographs obtained from these muscles not all the T-tubules are swollen suggests that only some of these links with the plasma membrane remain open under these conditions. Quantitative analysis of this effect is rendered very difficult by the irregularity of the swelling, which occurs primarily between the fibrils or at the edge of the circumfibrillar triads.

The open tubules may be those that become swollen by the inflowing isotonic fluid. The non-swollen tubules, which have an essentially normal appearance, are possibly those that have sealed off during the FMD treatment becoming, effectively,

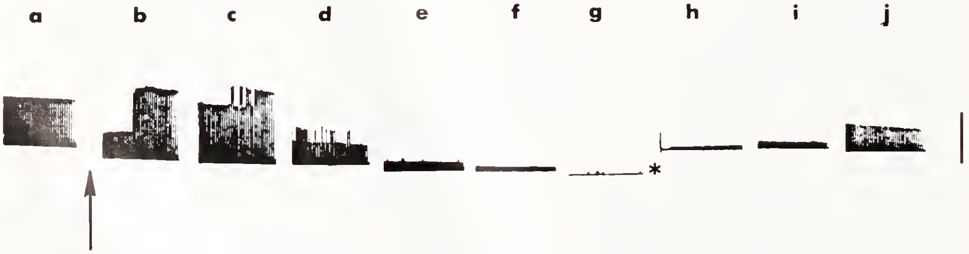

FIGURE 8. Time course of the blockade of contractility of a frog sartorius muscle immersed in 1.0 $M$ FMD and its partial recovery upon transfer to 1.0 $M$ EG (*). *a* records the control twitch tension developed in response to direct electrical stimuli at a frequency of 1 Hz. At arrow, FMD was added to the bath solution. *c, d, e* and *f* were recorded 3, 6, 12, and 24 min after. *f* illustrates the blockade after 32 min in FMD. *h, i* and *j* were recorded after placing the muscle in EG for 6, 12, and 24 min. Vertical calibration, 0.5 g; recording times, 1 min.

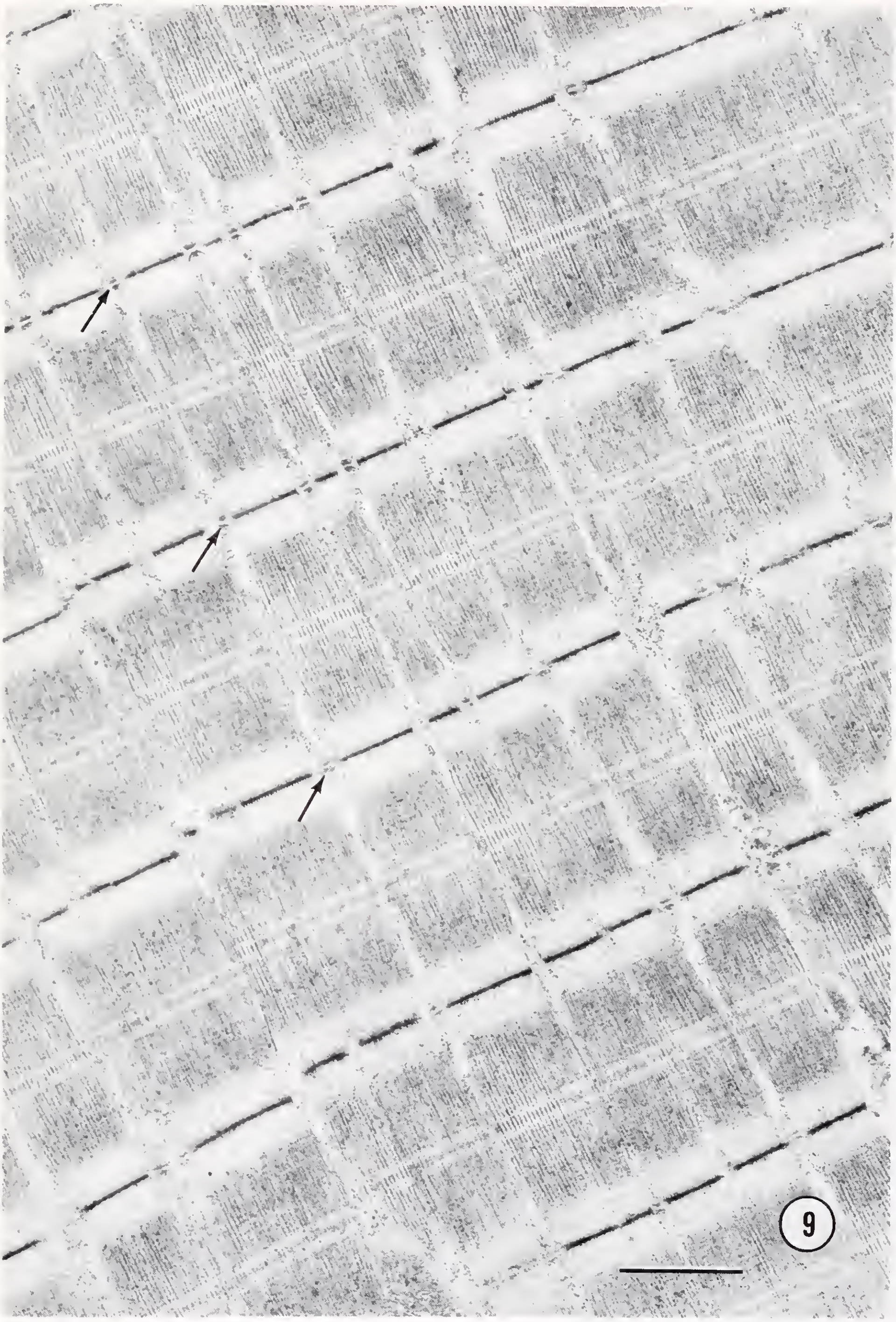

FIGURE 9. A survey field of sartorius muscle soaked sequentially in 1 *M* formamide and isosmotic EG prior to fixation in glutaraldehyde. The appearance of the myofibrils (in this instance, relaxed) and associated triad junctions (arrows) is essentially normal; in particular, the T-tubule swelling seen in Figures 5 and 6 is absent. Scale bar: 1 $\mu$m.

intracellular structures. In this respect, they would be similar to the terminal cisternae of the sarcoplasmic reticulum, which do not appear to be noticeably altered or swollen in any of the micrographs. Conversely, it is possible that swelling occurs only in sealed tubules, but this does not affect the functional interpretation.

Thus, the irreversibility of the uncoupling action of FMD on frog skeletal muscle transferred to normal Ringer's may be attributed both to the sealing of many of the T-tubules and to the swelling, and consequent loss of function, of others. In both instances, the normal triadic structure and/or function would be altered, resulting in complete loss of E-C coupling.

This conclusion is supported by the two above-mentioned observations: 1) In guinea pig ileum, a smooth muscle that lacks a tubular system, there is no obvious osmotic change when FMD-equilibrated strips are placed back in isotonic Krebs-Ringer's where the block of contractility is completely reversed. 2) When an osmotic shock is avoided, by transferring frog sartorius muscles to isosmotic EG solutions, there is a slow and partial recuperation of contractility suggesting that enough of the T-tubules remain open to permit effective E-C coupling.

We have observed changes in the after-potentials of spikes induced in muscles transferred to normal Ringer's after FMD-blockade which further suggest the occurrence of tubular disruption (Escalona de Motta *et al.*, 1982). This led us to propose that FMD exerts two separate effects on muscle contractility: a) a direct reversible inhibition, similar to that observed in guinea pig ileum, probably related to an interference with the activating action of $Ca^{2+}$ on the contractile machinery; and b) an irreversible effect occurring only when skeletal muscles equilibrated in hypertonic FMD solutions are suddenly brought back to normal saline.

The present observations emphasize that FMD has a direct inhibitory action on the E-C coupling process that reverses slowly when the amide is removed from the preparation, avoiding drastic osmotic changes. FMD must then be included among the permeant solutes suitable for uncoupling excitation from contraction. However, as reported earlier (Escalona de Motta *et al.*, 1982), compared to other agents in this category, FMD treatment is far more gentle and better preserves the electrical parameters of the muscle fibers.

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# RHYTHMS IN LARVAL RELEASE BY AN ESTUARINE CRAB (*RHITHROPANOPEUS HARRISII*)

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## ABSTRACT

Ovigerous females of the crab *Rhithropanopeus harrisii* were collected subtidally, and their rhythms in larval release monitored under constant conditions in the laboratory. Larvae from a single crab are generally released as a burst lasting less than 15 minutes. Larval release by crabs from an estuary lacking regular tides mainly occurs in the 2-h interval after sunset and is not related to coastal tides, which suggests a circadian rhythm. This rhythm can be entrained on an altered light-dark cycle. Larval release by crabs from an estuary with semi-diurnal tides begins at high tides and continues for 2 hours, suggesting a circatidal rhythm. Significantly more releases occur during the night. Crabs from the estuary without regular tides change from a circadian to a circatidal rhythm after being in the estuary with semi-diurnal tides. Alternatively, crabs from the estuary with semi-diurnal tides change to a circadian rhythm when exposed to a light-dark cycle and non-tidal conditions in the laboratory. Thus *R. harrisii* has both circadian and circatidal rhythms in larval release with the expressed rhythm dependent upon prior environmental conditions. Nighttime release may reduce predation, while release at high tide may minimize larval exposure to stressful, low salinity water.

## INTRODUCTION

Rhythms in reproductive activity and larval release are common among crustaceans. Timing may be related to lunar phase, time of day, and/or phase of the tide. Semilunar cycles are known for semi-terrestrial crabs (Gifford, 1962; Warner, 1967; Henning, 1975; Klassen, 1975; Saigusa and Hidaka, 1978; Seiple, 1979; Saigusa, 1981), intertidal fiddler crabs (von Hagen, 1970; Zucker, 1976, 1978; Christy, 1978; Wheeler, 1978), and subtidal stomatopods (Reaka, 1976). For the lobsters *Nephrops norvegicus* (Moller and Branford, 1979), *Homarus gammarus* (Ennis, 1973; Branford, 1978), and *H. americanus* (Ennis, 1975), larval release in the laboratory occurs shortly after dusk on a series of consecutive nights; no lunar or semilunar rhythm has been reported. *H. americanus* occasionally releases larvae during the day (Ennis, 1975).

Detailed laboratory studies of estuarine intertidal fiddler crabs indicate that females release their larvae within several hours after the time of the nocturnal high tide (DeCoursey, 1979; Bergin, 1981). As implied by DeCoursey (1979), precisely timed larval release may not be restricted to intertidal fiddler crabs but could also extend to other estuarine species.

This study was undertaken to examine larval release by the estuarine crab *Rhithropanopeus harrisii* which occurs from the very low intertidal zone into subtidal

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areas (Williams, 1965). Crabs from an estuary having pronounced semi-diurnal tides were compared to crabs from an estuary lacking regular tides. Experiments were designed to determine the presence of biological rhythms in larval release, the relationship of release time to environmental cycles, and the ability of the crab to change its rhythm under different environmental conditions.

## MATERIALS AND METHODS

Ovigerous female *Rhithropanopeus harrisii* (Gould) were collected from two coastal estuaries in North Carolina, the Neuse River (estuary A) and the Newport River (estuary B). Tides in estuary A are aperiodic (Roelofs and Bumpus, 1953). Physical factors such as salinity, water depth, and wave turbulence which usually vary with the tides, vary instead with wind direction and rain. In contrast, estuary B has regular semi-diurnal tides and periodic variation in tide-related hydrography (Cronin, 1982).

Crabs were obtained in wire mesh traps. In estuary A traps were placed at a depth of about 1 m on a gradually sloping bottom. Traps in estuary B were placed in an area having a relatively uniform depth of 3–4 m at high tide.

Ovigerous crabs were collected during the day, separated in the laboratory according to embryo development (based on yolk consumption and eye development), and placed in 20-cm diameter finger bowls containing water of the same salinity as at the collection site. The proper salinity was obtained by diluting sea water (filtered to remove particles larger than 5 $\mu$m) with distilled water. Crabs were either placed under constant conditions of temperature, salinity, and light, or entrained to a new LD cycle in an environmental chamber (Sherer Gillett Co., Model CEl 4-4). None were fed.

Larval release usually occurs during a specific interval in the LD or tidal cycle. The time was determined by intensively monitoring larval release over a designated 5-h sampling interval within the LD or tidal cycle. During this 5-h period, crabs were transferred every 15 min to a new 7.9-cm diameter finger bowl. At the end of the sampling period, the crabs were placed in 10.4-cm diameter finger bowls, and if eggs remained, the procedure was repeated at the next monitoring time, either 7.4 or 19 h later (see below).

The number of larvae released within each 15-min interval and between sampling intervals was recorded. Most larvae are released within 15 min, though a few commonly appeared in the intervals immediately preceding and following the peak. The mean time was calculated by multiplying the number of larvae released per 15 min by that interval, taking the sum of these products over all intervals, and dividing this sum by the total number of larvae. In this way a single 15-min interval was designated as the time of larval release. If a crab released the majority of its larvae during the period between the 5-h sampling periods, release was designated as occurring at "other times." About 12% of the crabs released bursts of larvae during two consecutive sampling periods. Using the above procedure a mean time was calculated for each release.

A chi-square test for goodness of fit was used to determine whether the number of releases during the intensive sampling time differed from an expected uniform rate throughout the solar day or over an entire tidal cycle. For these tests, the solar day included a sampling time and the preceding 19 h, while a 12.4-h tidal cycle encompassed the sampling time and the preceding 7.4 h. For crabs from estuary B, a chi-square test was used to determine any preference for releasing at day or nighttime high tides. A Kolmogorov-Smirnov goodness of fit test was used to determine if releasing was nonuniform throughout the intensive sampling time. Finally, linear regression analysis was used to estimate the period length of the rhythms by the population. For crabs, which release a burst of larvae during two consecutive sampling intervals, only the time of the first burst was used in this analysis. In this way each crab only contributed one time to the data. Larval release was monitored in five situations. The specific procedures for each situation are described in the next section.

## RESULTS

### *Estuary A: crabs from natural conditions*

In preliminary experiments larval release by crabs from estuary A was monitored at 2-h intervals under constant laboratory conditions for 3 days. Releases began just after sunset and continued for several hours. Releasing could be related to time of day or perhaps to tides, even though tides are considered aperiodic at the collection site.

To distinguish between these possibilities crabs were collected at weekly intervals for one month (May 16 to June 13, 1981). The tidal phase at dusk on the nearby coast alternated weekly between spring high tides and neap low tides. After collection and embryo staging, all crabs were maintained under room lights until the time of normal sunset when they were placed in constant low level light (photographic safelight containing a 15-W bulb and fitted with a Kodak OA filter; wavelength maximum = 573 nm, half band pass = 37 nm, intensity = $1.2 \times 10^{-2}$ W/m$^2$), and temperature (28 ± 1°C). A crab remained under constant conditions until it released its larvae or until 6 nights had elapsed and the experiment was terminated. Beginning 1 h before the time of the first sunset, all crabs with advanced embryos were transferred through the series of finger bowls. Other crabs were tested as their embryos matured.

At both collection times, each crab had embryos at one stage of development. However within the collected crabs, embryo development was not uniform, as all stages were observed. The number of crabs that released larvae in the laboratory within 6 nights of collection was similar at both collection times (high tide collection, n = 133; low tide collection, n = 110). These results suggest there is no lunar or semi-lunar cycle in larval release.

During spring high tides significantly more releases occurred during the sampling time on nights 1–5 (Fig. 1A; nights 1–4, $P < 0.005$; night 5, $P < 0.025$) than expected if releasing occurred uniformly throughout the solar day. Furthermore, releasing was not uniformly distributed within the 5-h sampling intervals on nights 1–4 and 6 (nights 1 and 6, $P < 0.01$; night 3, $P < 0.02$; nights 2 and 4, $P < 0.05$). Therefore larval release by the population occurred during a relatively short time within the 4-h interval after sunset.

Similarly, when low tides occurred during the evening the total number of releases during the intensive sampling time was greater than expected (Fig. 1B; nights 1–6, $P < 0.005$). Furthermore, releasing within the sampling time was nonuniform on nights 1, 3, 4, 5, and 6 (night 4, $P < 0.01$; night 6, $P < 0.02$; nights 1, 3, and 5, $P < 0.05$). Again larvae were released mainly within several hours after sunset.

There was no significant difference in the distribution of release times on specific nights during evening high and low tides (Mann-Whitney U test). (*i.e.*, comparison of nights 1, nights 2, *etc.*). To further compare the two situations, a regression was determined for the relationship of release time and night in constant conditions. Night 1 was excluded because release times may be influenced by initial adjustments

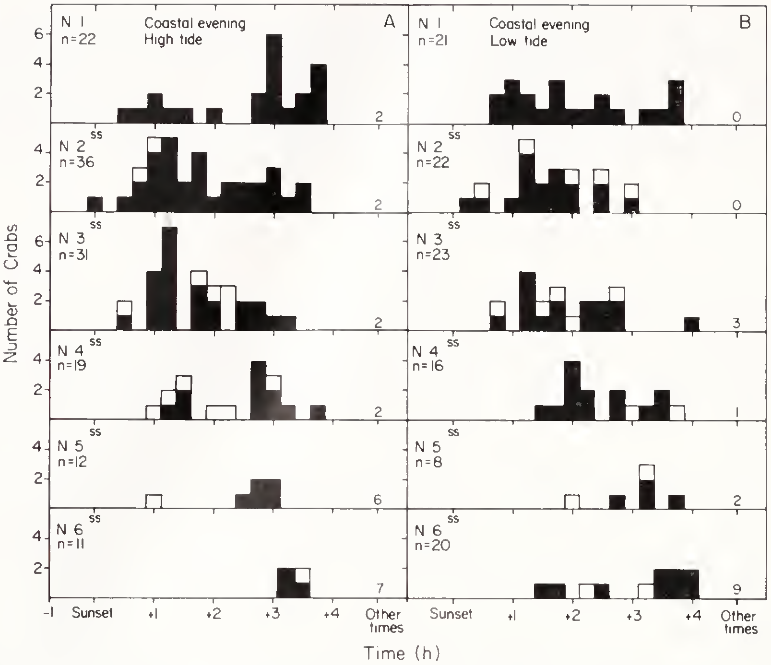

FIGURE 1. Number of crabs releasing larvae (ordinate) at times (abscissa) relative to sunset in estuary A. Crabs were under constant conditions in the laboratory and releasing was monitored during a 5-h interval on successive nights (N) when spring high tides (A) or neap low tides (B) occurred during the evening on the nearest coast. On N1 in A high tide occurred at about the time of sunset while low tide occurred at this time in B. The sample size (n) on each night is shown and "other times" indicates the number of releases at times other than the sampling time. For example the releases shown on N2 for "other times" indicate those that occurred between the end of the first and beginning of the second sampling interval. The second release of a crab is indicated by an open histogram.

to laboratory conditions. The slopes of the regression lines for releasing at both evening high and low tides were significantly different from zero (*t*-test; $P < 0.01$). When the two regressions were compared by an analysis of covariance, neither the slopes nor the intercepts were significantly different (F-test). These findings suggest release time is not related to coastal tides, and the data in Figures 1 and 2 were therefore combined for the following analysis.

Larval release predominantly occurred within a specific time interval on consecutive days in constant conditions, which suggests the crabs have an endogenous rhythm. The period of the rhythm of individual crabs can be estimated from the time between consecutive larval releases. Nineteen percent of the crabs released on two consecutive nights. The mean time between releases was 24 h (SE = 15 min; n = 25), when rounded off to the nearest 15-min interval.

In addition the period length of the population rhythm can be estimated by a regression analysis of the relationship between release time and night after placement in constant conditions (Fig. 2). Night 1 was not included, and only releases during the sampling interval were considered. On nights 5 and 6 the number of crabs releasing at times other than the intensive sampling time increased. Nevertheless these nights were included because a significantly ($P < 0.005$) greater number of crabs released in the 5-h sampling interval than predicted if releasing was uniform over the solar day. The slope of the regression line (Fig. 2) is significantly different

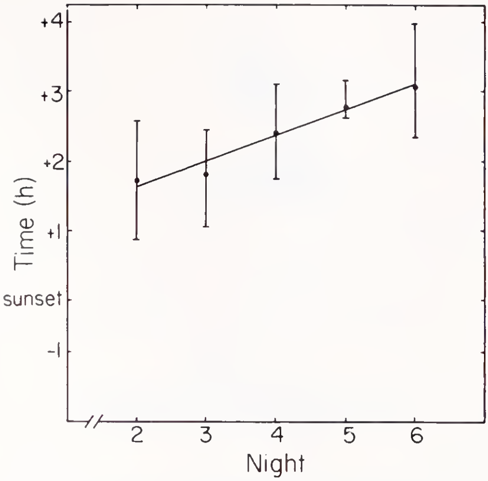

FIGURE 2. Regression of time of larval release (ordinate) for the combined data from Figures 1 and 2 on the consecutive nights in the laboratory (abscissa). The first release by all crabs within the intensive sampling interval was used for the analysis while the mean and standard deviation are shown on the figure.

from zero (*t*-test; $P < 0.001$), and predicts a time of 24 h 23 min between consecutive releases. Thus both the time between releases by a single crab and the regression analysis of the population indicate the free running period length is near 24 h. This implies the presence of a circadian rhythm in constant conditions in the laboratory and a daily rhythm in nature.

*Estuary B: crabs from natural conditions*

Larval release by these crabs occurs near the time of high tide (Cronin and Forward, 1982). To determine the precise relationship between release time and tide, crabs were collected at weekly intervals several hours before daytime high tide during July and August, 1980 and 1981. Since larval release was monitored in crabs collected in the same area at the same time of the year, data from the two years were pooled. Crabs were collected at a depth where daylight probably is not visible (see below); thus crabs were returned to the laboratory in opaque bottles and then sorted according to state of embryo development. The only assured times the crabs experienced light were the short intervals during removal from the traps and when the embryos were staged. All crabs were then placed under constant conditions and larval release monitored as described previously. The first sampling time began just after staging of embryos. For this tide, crabs with the most advanced embryos were monitored for 4 h beginning about 1 h before high tide in the field. On the following 6 tides, crabs with mature embryos were monitored from 2 h before high tide in the field to 3 h later.

Larval release by crabs from estuary B is not uniform (tides 1–7; $P < 0.005$) over a complete tidal cycle (Fig. 3). Ninety-three percent of all releases occurred near high tide during the sampling time. Within this interval, releasing was distributed uniformly except for tides 1 and 2 ($P < 0.01$), when most larvae were released in the 2-h interval after high tide.

Releasing occurred near the times of high tides, which suggests the crabs have a biological rhythm. At the individual level, fifteen percent of the crabs (n = 25)

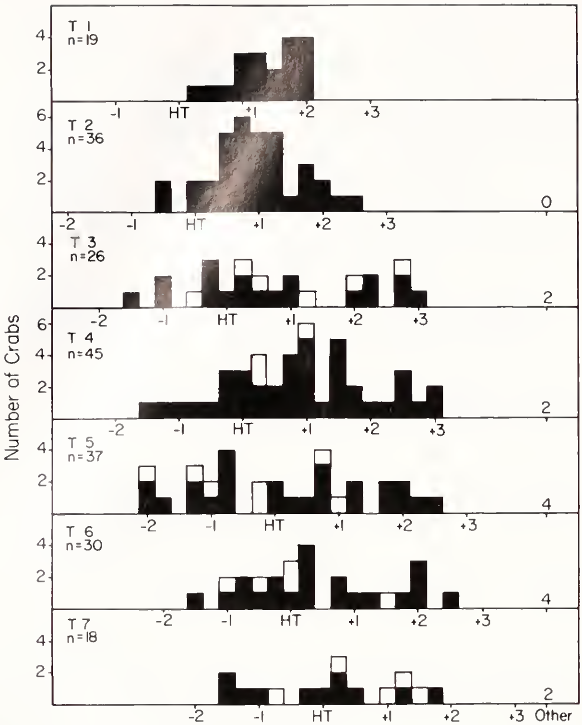

FIGURE 3. Number of crabs releasing larvae (ordinate) at times relative to high tide (HT) in estuary B. Larval release was monitored for a 5-h period around HT at the times of successive high tides (T) in the field (abscissa). The sample size (n) for each high tide is shown and "other times" indicates the number of releases at times other than the sampling interval. For example, the releases shown on T3 for "other times" are the number that occurred between the end of the second and beginning of the third sampling interval. The second release of a crab is indicated by an open histogram.

released larvae during successive high tides, while about 24 h elapsed between releases by one crab. The mean time between successive releases was 12 h 15 min (SE = 15 min) when rounded off to the nearest 15-min interval. The relationship between larval release by the population and tide (Fig. 4) is significant (*t*-test; $P < 0.001$). Furthermore, the slope of the regression line indicates the time between releases on consecutive tides is 12 h 12 min. Multiple releases by a single crab as well as the regression analysis of the population indicate that the free running period length is around 12 h 15 min. This implies the presence of a circatidal rhythm under constant conditions in the laboratory and a tidal rhythm in nature.

Although releases occurred on successive tides, it is possible that there is a day/night component in the rhythm. The average natural photoperiod throughout the experiments was 14 h light and 10 h dark. If releasing is independent of the light-dark cycle, then the predicted frequencies during the day and night sampling intervals would be 58 and 42%, respectively. The observed frequencies during the day and night are 48 and 52%. Releasing was not uniform during daytime and nighttime high tides (chi-square test, $P < 0.005$), as a significantly greater amount occurred near the time of nighttime high tides.

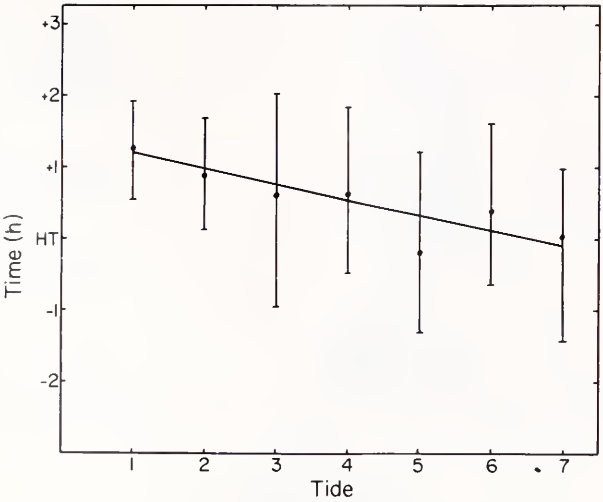

FIGURE 4. Regression of time (± SD) of larval release (ordinate) on the various tides (abscissa) for crabs from estuary B. The first release by all crabs within the intensive sampling interval was used for the analysis while the mean and standard deviation are shown on the figure.

## *Estuary A: entrainment to a LD cycle in the laboratory*

Two methods were used to determine if the apparent circadian rhythm of crabs from estuary A could be entrained by a light-dark cycle. In the first method, crabs were collected during the day and those with embryos in early stages of development were held at 27.0°C under a 14:10 LD cycle in the environmental chamber. The light intensity during the day phase was about 2.0 $W/m^2$ (cool white fluorescent lamps). The length of the photoperiod was similar to that in the field, but the beginning of the dark phase occurred 6 h before sunset. The crabs were maintained under these conditions for 5 days because preliminary experiments showed this duration was sufficient to reset the timing of the rhythm. Crabs were then placed under the same constant conditions as described above for crabs from estuary A. The method for monitoring release was also similar except that the sampling time began 1 h before the end of the light phase. Crabs were monitored for 3 days.

The time of larval release shifted with an altered LD cycle in the laboratory. When the time of "lights off" (laboratory sunset) occurred 6 h before the normal sunset in the field, the time of releasing shifted similarly after 5 days of entrainment (Fig. 5). Clearly, releasing was not uniform over the day, since it only occurred during the sampling time. Within this interval, releasing was also not uniform on all 3 nights ($P < 0.05$). Larval release began at the end of the light phase and continued for about the next 1.5 h. A regression analysis of the population release times was not performed because crabs were monitored only over 3 days. Nevertheless, at the individual level 13% of the crabs (n = 10) released larvae on consecutive nights. The mean time between releases was 24 h 15 min (SE = 15 min) when rounded off to the nearest 15-min interval, which approximates the suggested free running period length of population from the field (Fig. 2).

The second method involved monitoring larval release by crabs that were maintained under summer conditions during the winter in a laboratory habitat. Crabs

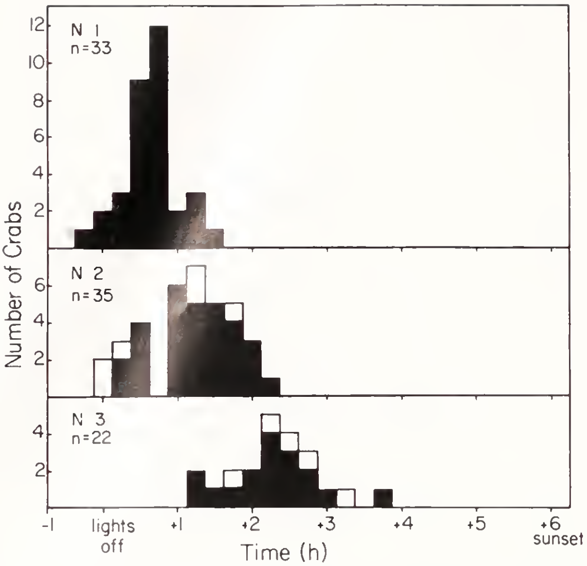

FIGURE 5. Number of crabs from estuary A releasing larvae (ordinate) at times (abscissa) relative to the end of the light phase. Crabs were placed on a 14:10 LD cycle in the laboratory for 5 days and then maintained under constant conditions. "Lights off" indicates the end of the light phase on the first night and "sunset" is the time of sunset in estuary A. Other symbols, as in Figure 1. No releases occurred at other times.

were held at 26.0°C in 9 ppt sea water, subjected to a summer photoperiod (15:9 LD cycle; cool white fluorescent lamps positioned over the tank; intensity = 7.5 W/m$^2$) and fed with Purina cat chow. Females became ovigerous in January and breeding continued through the spring. The method for monitoring releasing was identical to that for the previous crabs on an altered LD cycle. However during constant conditions the crabs were placed in the environmental cabinet (temperature, 26°C) having low intensity red light (6.5-W red incandescent lamp; wavelength output was greater than 600 nm; intensity about 0.3 W/m$^2$). Releasing was monitored for 3 days.

Winter crabs also exhibited a rhythm in larval release that was entrained to the altered LD cycle (Fig. 6A). Only 4% of the crabs did not release larvae in the sampling time which indicates releasing was nonuniform over the solar day ($P < 0.005$). On all 3 nights the release distribution was not uniform within the sampling interval ($P < 0.01$). Again releasing began at the end of the light phase and continued for about the next 1.5 h. Eleven percent of the crabs (n = 5) released larvae on consecutive nights, with a mean time between releases of 23 h 30 min (SE = 15 min) when rounded off to the nearest 15-min interval.

### *Estuary B: entrainment to a LD cycle in the laboratory*

To determine whether crabs from estuary B could change from an apparent circatidal to a circadian rhythm, crabs with embryos which would hatch between 6 and 9 days after capture were collected shortly before high tide and placed in the environmental chamber under a 14:10 LD cycle (cool white fluorescent lamps plus a 60-W incandescent bulb; intensity = 9.0 W/m$^2$) at 27°C. In order to separate daily and tidal influences, the time of the LD cycle was adjusted so that the dark phase

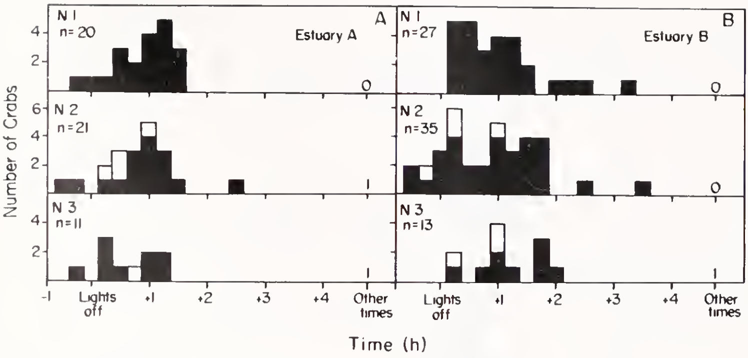

FIGURE 6. Larval release by crabs from estuary A (A) and estuary B (B) that reproduced during the winter. Crabs were maintained on a 15:9 LD cycle, and releasing was monitored under constant conditions. "Lights off" indicates the end of the light phase on the first night. Other symbols, as in Figure 1.

began at the predicted time of daytime low tide in the field 6 days after collection. After 6 or 7 days crabs were placed under constant conditions similar to those used for the tidal rhythm experiments with crabs from estuary B and larval release was monitored intensively for 5.5 h, beginning 1 h before the beginning of the dark phase. Preliminary experiments indicated that 6 days was the minimum time necessary for the crabs to change their rhythm. Releasing was monitored for only 3 days because the total time for embryonic development is about 10 days. Few crabs still had eggs at the end of the experiment.

Crabs released larvae during the 1.5-h interval after the end of the light phase (Fig. 7) rather than during the time following high tide in the field. Releasing was neither uniform during the solar day ($P < 0.05$) nor during the sampling interval for all nights ($P < 0.05$). Eight percent of the crabs ($n = 5$) released larvae on consecutive nights. The mean time between releases was 24 h (SE = 15 min).

To further establish that crabs from estuary B can develop a circadian rhythm, crabs were maintained over the winter in a habitat identical to that used for crabs from estuary A, and releasing was monitored using similar procedures. Larval release by winter crabs was also related to the LD cycle (Fig. 6B), as only 1% of the crabs did not release larvae during the intensive sampling interval (nonuniform releasing over the solar day, $P < 0.005$). Releases were nonuniform within the sampling interval on all nights ($P < 0.05$) with most releases occurring in the 1.5-h interval after the end of the light phase. Twelve percent of the crabs ($n = 8$) released larvae on consecutive nights. The mean time between releases was 23 h 30 min (SE = 15 min), which corresponds to the time observed for crabs from estuary A under similar conditions (Fig. 6A).

### *Estuary A: entrainment to natural tidal conditions*

A final experiment determined whether crabs from estuary A could change from an apparent circadian to a circatidal rhythm in larval release. Since the environmental cycles which entrain the tidal rhythms are unknown, male and nonovigerous female crabs from estuary A were placed in plastic boxes containing mollusk shells in traps at the collection site in estuary B. Holes in each box permitted water flow through the box but prevented the crabs from escaping. Beginning 10 days after crabs were translocated to estuary B, ovigerous females were collected at weekly

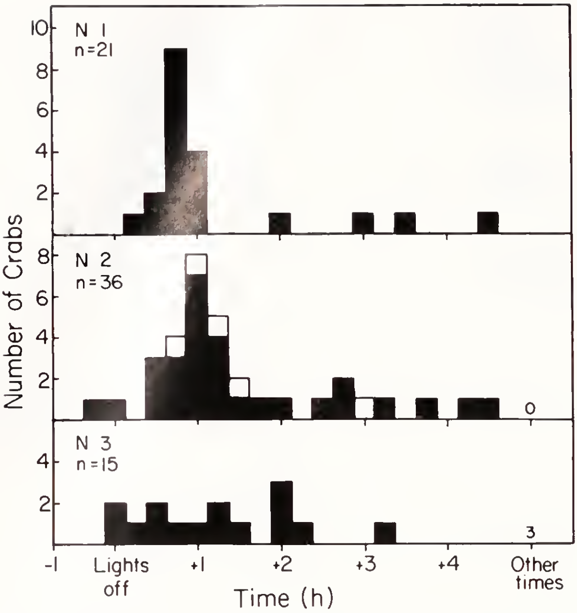

FIGURE 7. Number of releases (ordinate) at times (abscissa) relative to the end of the light phase for crabs from estuary B which were placed on a 14:10 LD cycle in the laboratory for 6 or 7 days and then maintained under constant conditions. "Lights off" indicates the end of the light phase on the first night. Other symbols, as in Figure 1.

intervals for a month. The crabs were collected several hours before daytime high tide, and larval release measured as described for crabs from estuary B (tidal experiments).

When crabs were transferred from estuary A to estuary B, they developed an apparent circatidal rhythm (Fig. 8). Eighty-four percent of all releases occurred during the 5-h sampling time around high tide (nonuniform releasing over the tidal cycle, $P < 0.005$), though releasing was uniform within this interval on all tides. There was no significant preference for day or night high tides. Three of the crabs released larvae during consecutive sampling times.

## DISCUSSION

The crab *Rhithropanopeus harrisii* shows rhythms in larval release that are related to environmental cycles in the habitat where it lives. At the collection site in estuary A, crabs experience the natural light-dark cycle and a diel temperature cycle (unpublished observations). Tides in this area are aperiodic (Roelofs and Bumpus, 1953) even though tidal currents may occur in the estuary (Knowles, 1975). In the experiments, larval releases of crabs from this estuary were not related to tides but rather began at sunset and continued for about 2 h (Fig. 1). The observed time of larval release does not reflect the monitoring regime or handling, since the identical release pattern was observed in preliminary experiments when crabs were handled every 2 h for 3 days.

The relationship between releasing and the time of sunset suggests the presence of a circadian rhythm. A circadian rhythm is normally defined as an endogenous rhythm which persists for at least 5–10 cycles in a single individual under constant

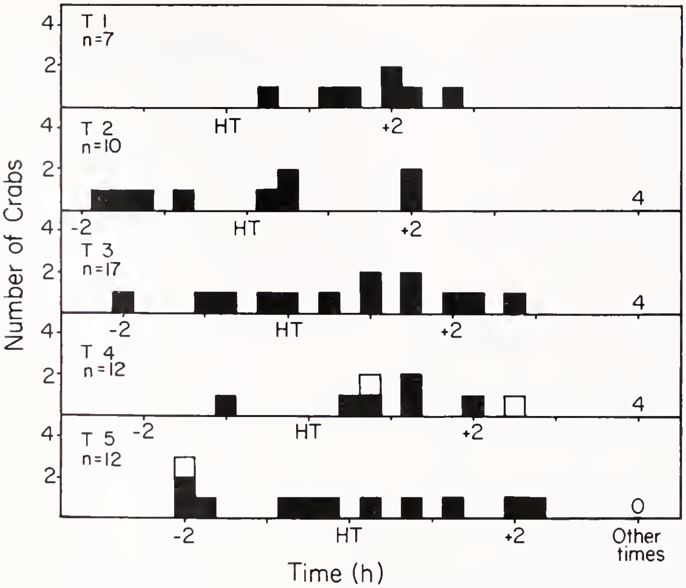

FIGURE 8. Larval release by crabs from estuary A that were transplanted to estuary B. Notations as in Figure 3.

conditions and which has a free running period close to but not exactly 24 h. Larval release occurs either as a single event in one crab or, at most, two events on consecutive nights. Thus the criteria of persistence of the rhythm in a single individual for 5–10 cycles cannot be fulfilled. Nevertheless, the rhythmic release of larvae by a population of crabs does persist under constant conditions in the laboratory for at least 6 diel cycles (Fig. 1). Also, the time between consecutive releases by a single crab and the regression analysis of population release times (Fig. 2) indicate free running period lengths of nearly 24 h. These considerations suggest that there is a circadian rhythm in larval release by individual crabs in constant conditions in the laboratory which is observed as rhythmic releases by the population within a specific time interval on successive days. Furthermore, these results indicate that the population has a daily rhythm in larval release in nature.

In contrast, crabs from an estuary with semi-diurnal tides had the greatest number of releases in the 2-h interval after high tide (Fig. 3). This pattern is not due to the sampling regime, since similar results were obtained in preliminary experiments when the crabs were sampled every 2 h for 6 days (Cronin and Forward, 1982). These results suggest the presence of a circatidal rhythm. The rhythmic release of larvae by the population persisted for seven tidal cycles under constant conditions and had a free running period of about 12 h 15 min (Fig. 4) as did successive releases from a single crab. In nature the population would have a tidal rhythm in larval release.

These crabs also had a significantly greater number of larval releases at night, although a considerable number of releases occurred during daytime high tides. The absence of a strong preference for day or night may be due to environmental conditions. The crabs were collected in traps at a depth of 3–4 m. Here they would be exposed to pronounced tidal changes in current flow, depth, and salinity (Cronin, 1982), but it is unlikely that they would sense a diel LD cycle. This prediction is based upon the rapid attenuation of light in the estuary and assumes that the adults have the same spectral (Forward and Cronin, 1979) and intensity sensitivity (Cronin, 1979) as their larvae. Since the crabs can move from shallow to deep areas, we were probably sampling crabs which had and had not been exposed recently to the natural LD cycle. This may explain the weak preference for night in our results.

The crabs change both the time of larval release and the length of the free running period when exposed to different environmental cycles. Crabs from estuary A altered the time of releasing when entrained to a new LD cycle in the laboratory (Figs. 5 and 6A). Under a LD cycle and nontidal conditions in the laboratory, crabs from estuary B developed a circadian rhythm (Figs. 6B and 7). All crabs (estuaries A and B) that were entrained to a LD cycle in the laboratory, began larval release just after the end of the light phase and continued for about 2 hours. Releasing occurred at the same time for field-captured animals (Fig. 1), which suggests the LD cycle is the normal zeitgeber in the field.

A circatidal rhythm was induced in crabs from estuary A by placing them in estuary B (Fig. 8), but the zeitgeber remains unknown. The phase of the rhythm was similar to that for crabs living in estuary B. Interestingly, these crabs had a pronounced daily rhythm in their original habitat, yet lacked a day/night preference after exposure to conditions in estuary B. The transported crabs were placed in boxes at 3–4 m depth with no possibility of movement to shallower depths. It is unlikely the crabs experienced a LD cycle at this depth, which may explain the absence of a preference for larval release at night.

The crabs from both estuaries have the capability of showing both circatidal and circadian rhythms after exposure to different environmental conditions. Larval release by *R. harrisii* is not the only example of this flexibility. For example, this was also found in activity rhythms of the crabs *Carcinus maenas* (Naylor, 1958, 1960) and *Uca tangeri* (Altevogt, 1959) and in vertical migration patterns of *R. harrisii* larvae (Cronin and Forward, 1979). Of particular interest, however, is the time of release with respect to the tidal and diel LD cycles, since larval release could be most adaptive at a particular time of day or phase of the tide.

When not exposed to periodic tides, *R. harrisii* shows a daily rhythm with releasing occurring primarily in the 2-h interval after sunset. Larval release at night is commonly observed in laboratory studies of crustaceans such as lobsters (Pandian, 1970; Ennis, 1973, 1975; Branford, 1978; Moller and Branford, 1979), fiddler crabs (DeCoursey, 1979, 1981; Bergin, 1981), and the prawn *Macrobrachium idae* (Pandian and Katre, 1972). Nighttime release has been observed in field studies of fiddler crabs (Hyman, 1922; Christy, 1978; DeCoursey, 1981; Stancyk and Christy, 1981), pebble crabs (Knudsen, 1960), *Cardisoma guamhumi* (Gifford, 1962), *Aratus pisoni* (Warner, 1967), *Birgus latro* (Reese and Kinzie, 1968) and various *Sesarma* species (Saigusa, 1981). This suggests nocturnal larval release has a common functional advantage, which is probably avoidance of predators on larvae and adults which visually sight and actively pursue their prey (Ennis, 1975; Branford, 1978; DeCoursey, 1979; Bergin, 1981).

Larval release by *R. harrisii* from a tidal area occurs primarily in the 2-h interval after the time of high tides. In other detailed studies of crustaceans from an estuarine tidal area, fiddler crabs (DeCoursey, 1979, 1981; Bergin, 1981) and *Sesarma* sp. (Saigusa, 1981) had similar times of larval release. Since releases frequently occur near high tide, this again suggests a common functional advantage.

In estuaries, larvae encounter the problems of transport and of survival in a highly variable environment. By entering the water column near the time of high tide, subsequent horizontal movements are seaward as the tide recedes. This would tend to favor larval dispersal. Although this explanation is appropriate for fiddler crabs (Wheeler, 1978; Christy, 1978; Bergin, 1981; Stancyk and Christy, 1981) and *Sesarma* sp. (Saigusa and Hidaka, 1978; Saigusa, 1981), it is unlikely that the time of release by *R. harrisii* aids dispersal, since field studies (Bousfield, 1955; Pinschmidt, 1963; Sandifer, 1973, 1975; Cronin, 1982) indicate that massive seaward transport of larvae does not occur, and that all larval stages are retained in the area of the adult population (Cronin, 1982).

A more reasonable hypothesis is that larval release near the time of high tide functions as adaptation to avoid stressful or even lethal salinity conditions. Estuarine benthic crabs are exposed to changes in salinity over a tidal cycle, with the upper value rarely exceeding 35 ppt. Salinity tolerances of larvae from estuarine crabs are usually sufficient to cope with the maximum salinity values they are likely to encounter. For example, successful larval development of *R. harrisii* occurs between 2.5 and 40 ppt (Costlow *et al.*, 1966). The real tolerance problem is low salinity water, which would be experienced at low tide. Since salinity is potentially highest and thereby least stressful around high tide, this would be an appropriate time for estuarine crabs to release their larvae.

Both von Hagen (1970) and Saigusa (1981) also consider the timing of larval release to be related to salinity tolerance. They both suggest that if larvae are released around the time of spring high tide, the subsequent ebb would transport them towards the ocean where they would encounter less stressful, high salinity water. Since *R. harrisii* larvae are not transported to the ocean, larval survival may depend upon exposure to the highest possible salinity at the time of release.

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# GROWTH AND REGENERATION PATTERNS IN THE FIDDLER CRAB, *UCA PUGILATOR*

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## ABSTRACT

The fiddler crab, *Uca pugilator*, will survive several intermolt cycles in the laboratory, but the cycles are irregular. Variations in cycles are due to variations in the length of stage $C_4$. The transition from $C_4$ to D in intact crabs does not seem to be due to environmental clues because crabs kept in constant conditions for long periods of time continue to have extremely variable intermolt cycles.

Multiple autotomy triggers the onset of proecdysis and a post-autotomy intermolt cycle that is significantly shorter than controls. Multiple autotomy-induced proecdysis is divided into two phases: the "reset event" is independent of the eyestalks, while the "proecdysial program" is normally under their control. Loss of a cheliped is more effective in initiating a reset event than is loss of a single walking leg.

Eyestalk removal forces crabs into proecdysis. If crabs are in early proecdysis (stage $D_0$) at eyestalk removal, the proecdysial period is accelerated. Eyestalk removal results in large increases in size at ecdysis which can be blocked by multiple autotomy. Ecdysis does not always result in growth. Molting in *Uca* may result only in regeneration of missing limbs. Crabs regenerating a number of limbs may actually become smaller at molt.

## INTRODUCTION

Ecdysis of the calcified exoskeleton is the end point of a combination of physiological processes used by decapod crustaceans to achieve both general body growth and regeneration of appendages. Implicit in this statement is the assumption that the controls of ecdysis, growth, and regeneration are intimately linked and finely coordinated. Ecdysis and regeneration can be induced during non-growth periods by removal of the eyestalks or of many appendages. The former method induces ecdysis through removal of inhibitory neurosecretory centers in the eyestalk (the x-organ and sinus gland). The removal of the inhibitory centers usually causes a premature ecdysis. The second type of molt induction (called multiple autotomy) is more complicated and is thought to involve a "resetting" of the physiological processes that culminate in regeneration and ecdysis (Skinner and Graham, 1972).

The fiddler crab, *Uca pugilator*, is a durable and exceptional laboratory animal. One of the most remarkable features of these hardy little crabs is the single, large cheliped of the male (from which this entire group gets its common name). This cheliped (or claw) is often longer than the entire carapace of the crab. One third of the wet weight of a male crab may be due to the cheliped. The cheliped is very important in social and reproductive behavior of these crabs (Crane, 1975).

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Abbreviations: $C_4$, intermolt period of molt cycle; D, proecdysial period of molt cycle; E, ecdysis; ER, experimental growth rate; MA, multiple autotomy (including cheliped); MA-CI, multiple autotomy (cheliped intact); $R_3$, right third walking leg; R-value, regeneration index value for $R_3$.

The fiddler crab has been used by many investigators in various physiological and endocrinological studies (Abramowitz and Abramowitz, 1940; Guyselman, 1953; Passano, 1960; Vernberg and O'Hara, 1972; Skinner and Graham, 1972; Fingerman and Fingerman, 1974; Weis, 1976, 1977a,b). Relatively little has been reported, however, concerning growth and molt cycles of intact animals under constant laboratory conditions. This paper describes the molting cycles in intact fiddler crabs kept in constant environmental conditions and compares these "normal" cycles to autotomy-induced and eyestalk removal-induced cycles.

This paper includes observations on: (1) the effect of autotomy of various numbers of limbs upon the "reset" and duration of the intermolt cycle and growth patterns of the carapace and limbs; (2) the influence of autotomy of the large cheliped upon the intermolt cycle; (3) the effects of autotomy and eyestalk removal upon intermolt cycles subsequent to the induced cycles. The seemingly contradictory effects of multiple autotomy upon eyed and eyestalkless crabs has been investigated and a modified model for autotomy-induced proecdysis is proposed.

## MATERIALS AND METHODS

Male specimens of the fiddler crab, *Uca pugilator*, were obtained from the Gulf Specimen Company of Panacea, Florida. Shipments were received throughout the year. Upon arrival in the laboratory, the crabs were forced to autotomize the right third ($R_3$) walking leg (the fourth pereiopod) by pinching the merus with forceps. Individual crabs were kept in transparent plastic boxes (28 cm × 17.5 cm × 13.5 cm) with a small amount of artificial sea water (Instant Ocean, Aquarium Systems, Inc., Menton, Ohio). Crabs were kept in environmental chambers maintained at 23°C with 12 hours of illumination each day beginning at 6:00 AM. The crabs were fed oatmeal once a week and allowed to feed overnight. The water in the boxes was changed the following day. Animals were checked daily for molts. Crabs were allowed to acclimate in the laboratory at constant environmental conditions for at least two weeks prior to being used in any experiment.

The carapace width of each animal was measured with a vernier caliper (Moderntools, MT-9). The regenerating right third walking leg was measured every other day with the aid of an ocular micrometer in a dissecting microscope. In order to compare limbs from crabs of different carapace size, the length of a regenerating limb bud was converted to a Regeneration Index (Bliss, 1956).

$$\text{Regeneration Index (R-value)} = \frac{\text{length of limb bud (in mm)}}{\text{carapace width (in mm)}} \times 100$$

Subdivisions or stages of intermolt cycles were assigned as per Skinner (1962, after Drach, 1939).

The length of the large cheliped was also measured with the vernier caliper. This measurement is the linear distance from the notch at the base of propodus (at the point of articulation with the carpus) to the tip of the dactylus. The size of the cheliped (in mm) was divided by the carapace width (in mm) and this number is called the "Cheliped/Carapace Ratio" (C/C Ratio).

Following the emergence of the right third limb bud, multiple autotomy of additional walking legs and/or the large cheliped was induced as described above for the right third walking leg.

Eyestalks were removed by cutting the articulating membrane with a pair of dissecting scissors. Prior to eyestalk removal, animals were anesthetized by cooling at 4°C for 10 to 20 minutes.

Growth rates (ER's) of regenerating limb buds were calculated as previously described (Bliss and Hopkins, 1974): $R_3$ values are plotted against time (in days). For two consecutive $R_3$ values, the slope of the line connecting the points is taken as the experimental growth rate (ER) for the limb. The slope of the line is the arc angle of the sloped line relative to the horizontal. ER's were calculated for every day of an intermolt cycle. The average ER is the mean of those daily ER's.

Water content of chelipeds and walking legs was determined by blotting and weighing the limb immediately after removal, desiccating in a drying oven for four to six days, then weighing again. The difference in weight was taken as the water content of the limb. Protein content of chelipeds and walking legs was determined by grinding the desiccated limbs in ice cold 5% trichloroacetic acid (TCA) in a chilled mortar and pestle. The solution was centrifuged at 4°C and 10,000 × *g* for 20 minutes. The pellet was re-extracted with successive extractions in 80% and 100% ethanol, chloroform:ether (2:1 vol:vol), and ethyl ether. The pellet was resuspended in distilled water and the amount of protein determined by the method of Lowry *et al.* (1951) using bovine serum albumin (Sigma Chemical Co.) as the standard.

Statistical analysis of the data was handled as follows: means were determined and the homogeneity of variances was tested using the $F_{max}$ test (Sokal and Rohlf, 1969, p. 370). If the assumptions for normality were met, analyses were done using standard analysis of variance. However, if the assumptions of analysis of variance were not met, analogous non-parametric methods (Mann-Whitney U-test and Wilcoxon two-sample test) were used.

## RESULTS

### *Intermolt cycles in control animals*

The duration of intermolt cycles in intact crabs varies from crab to crab and from cycle to cycle. When maintained under the constant laboratory holding conditions described above, *Uca pugilator* can successfully complete as many as six intermolt cycles (Table I and Fig. 1a). The durations of these cycles range from 25 to 171 days. For crabs kept in the lab over three months, the range is 34 to 136 days. Animals maintained under constant environmental conditions for several months show some reduction in the mean duration of the intermolt cycles. The eventual clustering around a mean intermolt cycle of 70 days (Table I) is the result of a reduction in the number of extremely long intermolt cycles. The number of shorter cycles is unaffected.

After being held at constant conditions for several months, however, individual crabs continue to molt independently of one another: there are no "waves" of molting. The pattern of variable intermolt durations differs from one crab to another. An individual crab may take 125 days to complete one intermolt cycle and complete the next cycle in less than 50 days. Another crab in identical holding conditions may have two very long (or very short) successive intermolt cycles (Fig. 1a). The duration of a single intermolt cycle is never a prediction of the duration of subsequent cycles.

In *Uca*, the proecdysial period of any intermolt cycle requires about 27 days (Table II). This is true for crabs missing only one limb and for crabs missing eight limbs. Crabs that are destalked during $C_4$ (see Drach, 1939) take 26.6 days to reach ecdysis. Thus, the variations observed in intermolt cycle lengths represent variations in the duration of stage $C_4$ rather than in stage D.

These differences between cycle durations of crabs that have been in the lab under identical conditions are, in part, due to the variations in the sizes of the

TABLE I

*Number of days (mean ± standard error of the mean) from initial event (either autotomy of a single $R_3$ walking leg, multiple autotomy or eyestalk removal) to the first ecdysis in the lab.*

| | Controls (Lacking a single $R_3$) | | Multiple autotomy: 8 Walking legs (MA-CI) Cheliped Intact | | Multiple autotomy: 7 Walking legs + cheliped (MA) | | Eyestalkless | |
|---|---|---|---|---|---|---|---|---|
| | Number of days (±SEM) | n | Number of days (±SEM) | n | Number of days (±SEM) | n | Number of days (±SEM) | n |
| Initial event to Ecdysis 1 | 98.2 (±5.2) | 75 | 26.0 (±1.1) | 23 | 32.4 (±0.9) | 89 | 22.7 (±0.8) | 145 |
| Ecdysis 1 to Ecdysis 2 | 85.0 (±4.2) | 53 | 58.4 (±8.0) | 11 | 67.0 (±3.9) | 54 | 27.4 (±1.2) | 28 |
| Ecdysis 2 to Ecdysis 3 | 69.4 (±5.0) | 27 | 79.3 (±19.0) | 8 | 64.5 (±6.4) | 19 | 28.0 (±1.4) | 2 |
| Ecdysis 3 to Ecdysis 4 | 70.2 (±7.3) | 17 | 92.2 (±15.2) | 6 | 67.8 (±7.9) | 9 | | |
| Ecdysis 4 to Ecdysis 5 | 68.8 (±7.8) | 10 | 93.2 (±14.5) | 6 | 40.5 (±6.9) | 5 | | |
| Ecdysis 5 to Ecdysis 6 | 76.4 (±5.8) | 10 | 87.7 (±7.4) | 3 | 99.3 (±40.3) | 4 | | |

The mean number of days (±SEM) for subsequent ecdyses is also given. The number of crabs in each group is given as "n."

animals. When the duration of three subsequent intermolt cycles is plotted against the initial carapace width of the animal, a correlation of 0.43 is seen. (This correlation is significant at $P < 0.01$.) For example, a specific animal of carapace width 16.85 mm took 532 days to complete three intermolt cycles of varying durations. Whereas, a smaller crab, carapace width 14.70 mm, took only 220 days to complete three cycles. The pattern of alternating short and long intermolt cycles, however, remains the same in large and in small crabs.

### *Intermolt cycles following autotomy*

Autotomy of a single walking leg does not markedly affect the duration of the intermolt cycle (Fig. 1a). Therefore, crabs missing only one limb are referred to as "normal" or "controls" throughout this report.

The duration (and variance) from autotomy to ecdysis decreases as the regeneration load is increased (Fig. 2 and Table III). It continues to decrease until the load reaches 7 to 8 mg of protein. The regeneration load for an animal is calculated from the total amount of protein extracted from newly regenerated limbs following ecdysis.

Multiple autotomy during intermolt cycle stage $C_4$ significantly hastens the next ecdysis (Fig. 1b, Tables I and II). If eight walking legs are autotomized simultaneously and the cheliped left intact (MA-CI), the length of time from autotomy (= initial event) to the induced ecdysis is significantly shortened when compared to controls (Table I). However, the addition of the large, muscular claw to the regeneration load (MA) results in a period that is significantly longer ($P < 0.001$) than the comparable period in crabs missing only eight walking legs (Tables I and II).

The influence of an autotomized cheliped upon the induction of the proecdysial period (intermolt stage D) seems to be quantitatively different from the influence of the autotomy of a single walking leg. Autotomy of four walking legs shortens the time from autotomy to ecdysis when compared to controls (Table III). However, autotomy of three walking legs and the cheliped results in a significantly faster onset of ecdysis. Multiple autotomy of seven walking legs plus the cheliped (MA) is more effective in prolonging the late proecdysial period ($D_1$) than multiple autotomy of eight legs only. MA prolonged late proecdysis (stage $D_1$) in 11 out of 13 animals, while MA-CI was effective in prolonging $D_1$ in only three out of eight animals (Table II).

Multiple autotomy also has a pronounced effect on the second post-autotomy intermolt cycle (Fig. 1b and Table I). Not only is the immediately induced cycle affected by MA and MA-CI but also the second post-autotomy intermolt cycle is significantly shorter than that of the controls (Table I).

### *Intermolt cycles following eyestalk removal*

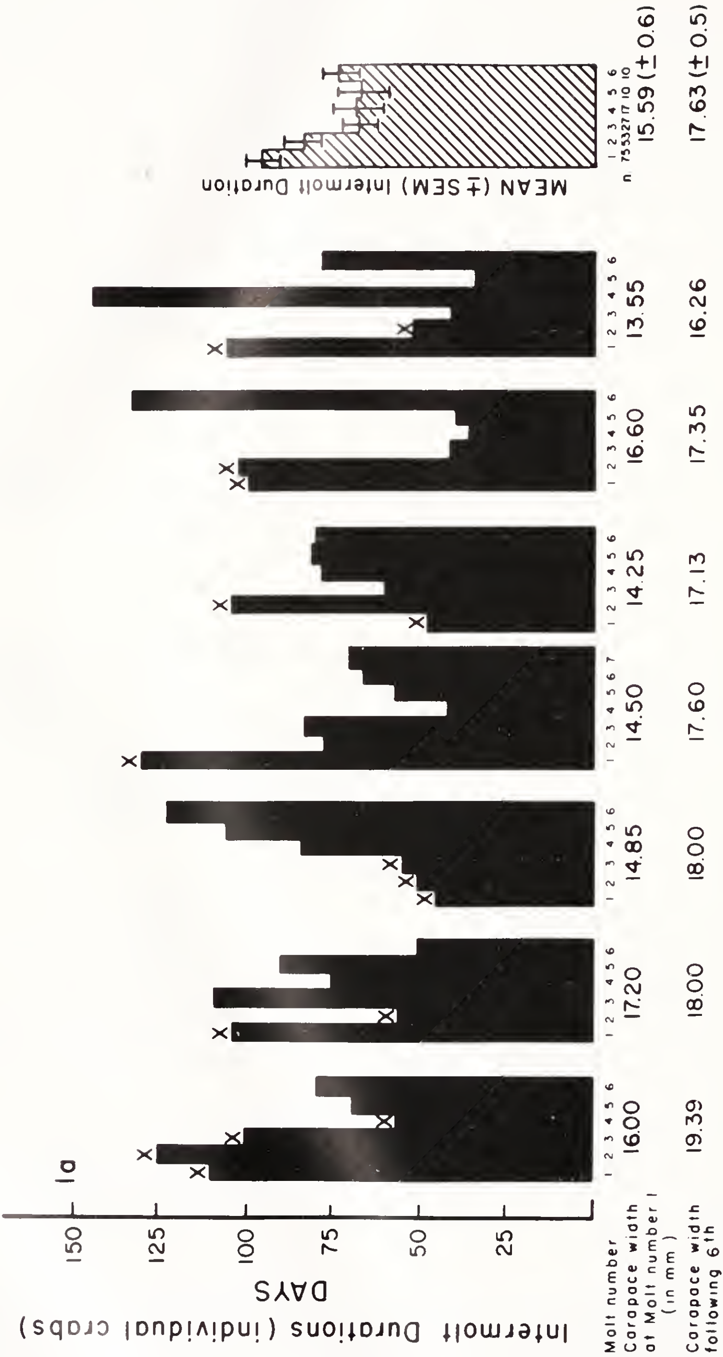
Removal of eyestalks hastens ecdysis (Tables I and II). When eyestalks are removed from crabs that have spontaneously entered proecdysis (stage $D_0$), the proecdysial period is shortened from 27.1 days to 18.7 days (Table II). Eyestalk removal from crabs in late proecdysis (stage $D_1$) reduces that period from 11.7 days to 4.8 days. Thus, it appears that the eyestalks continue to exert some inhibitory control during most of the proecdysial period. About 20% of eyestalkless *Uca* will live through a second molt cycle. These crabs molt within 28 days of the first ecdysis (Table I).

Multiple autotomy of seven legs and cheliped (MA) after eyestalk removal significantly prolongs the time from eyestalk ablation to ecdysis (Table IV). However, the number of days from MA to ecdysis (E) is less than comparable periods induced by MA in intact animals (Table IV). In fact, the time from MA to ecdysis in eyestalkless crabs is very close to (and statistically indistinguishable from) the time from eyestalk removal to ecdysis of otherwise untreated crabs (Table IV). Thus, MA in eyestalkless crabs may reset the proecdysial period but does not have any effect on the duration of the proecdysial period that follows.

Multiple autotomy during late proecdysis in eyestalkless crabs does not reset and actually speeds the proecdysial period. These crabs molt more quickly than do eyestalkless controls and they do not regenerate any of the newly autotomized limbs (Table IV).

### *Growth patterns in control animals*

*Regeneration of walking legs.* The averaged growth pattern of several right third walking limb buds is illustrated in Figure 3 (solid circles and solid line). The first event in limb regeneration is emergence of a limb bud papilla through the scar tissue that covers the coxal stump. The time between autotomy of a single limb and


1a
Intermolt Durations (individual crabs)
DAYS
150
125
100
75
50
25
Molt number
Carapace width at Molt number 1 (in mm.)
Carapace width following 6th Molt (in mm.)
1 2 3 4 5 6
16.00
19.39
1 2 3 4 5 6
17.20
18.00
1 2 3 4 5 6
14.85
18.00
1 2 3 4 5 6 7
14.50
17.60
1 2 3 4 5 6
14.25
17.13
1 2 3 4 5 6
16.60
17.35
1 2 3 4 5 6
13.55
16.26
MEAN (± SEM) Intermolt Duration
1 2 3 4 5 6
n 7 5 5 3 2 7 17 10 10
15.59 (± 0.6)
17.63 (± 0.5)

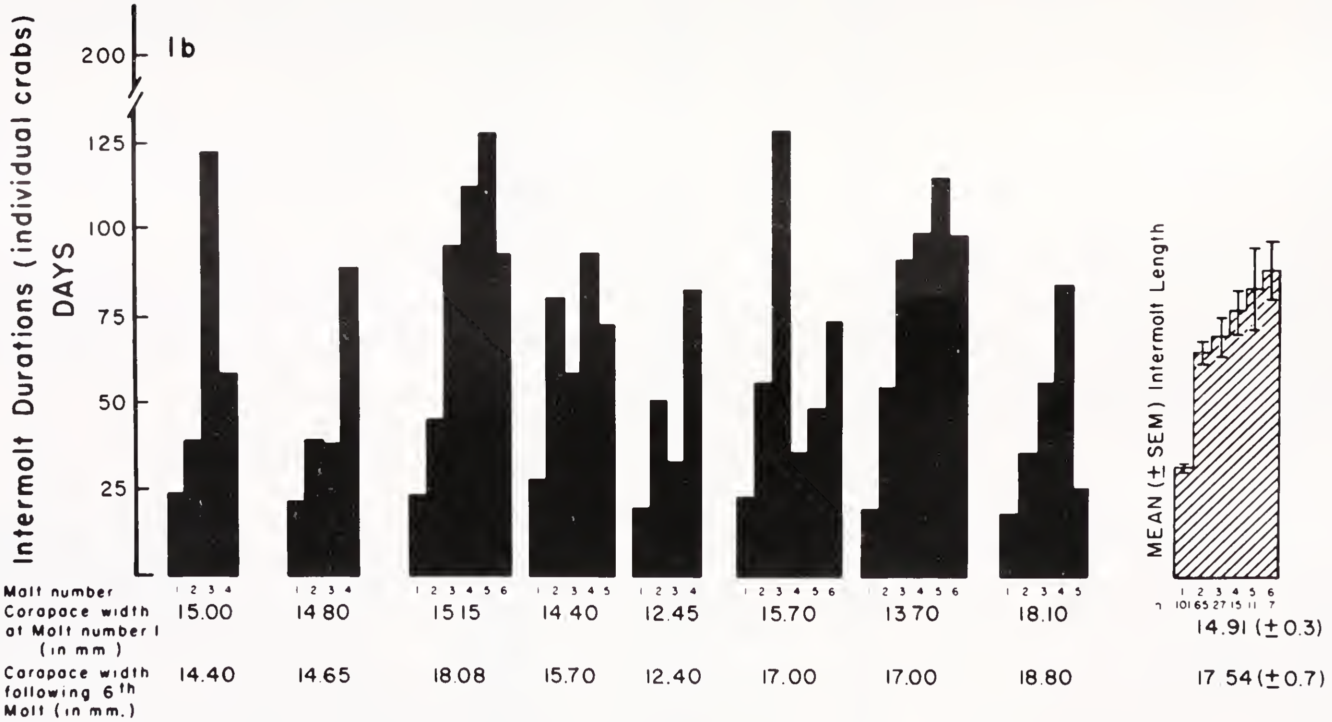


FIGURE 1. Intermolt cycle durations for selected individual crabs. (1a.) Control crabs (black bars) missing one $R_3$ walking leg (X) or missing no limbs (no X over bar). Means (± standard error of the mean) for large sample size (n) are given on right (lined bars). (1b.) Intermolt cycle durations of crabs missing seven walking legs plus cheliped (MA) or eight walking legs (MA-CI) prior to molt number one. Means (±SEM) for large combined samples (MA + MA-CI) are given on right (lined bars).

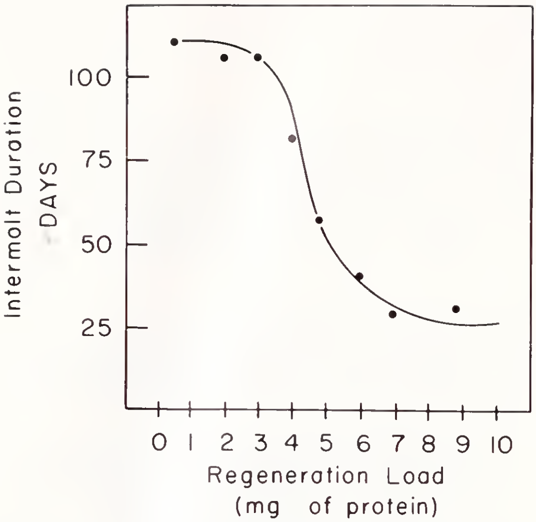


FIGURE 2. Intermolt duration (in days) as a function of regeneration load (= sum of extractable protein in mg from all regenerated limbs following ecdysis). Each point represents the mean of at least ten animals.

emergence of the papilla is quite variable in control crabs (Table III). Limb bud emergence in controls takes an average of 47% of the total cycle regardless of the length of the ensuing intermolt cycle.

The simultaneous loss of two or four walking legs hastens the emergence of all limb buds and significantly reduces the variances of emergence time (Table III).

TABLE II

*The effects of multiple autotomy of eight walking legs with cheliped left intact (MA-CI), multiple autotomy of seven walking legs plus cheliped (MA), and eyestalk removal (ES) at intermolt cycle stages $C_4$, $D_0$, and $D_1$ on the mean number of days from treatment (T) to ecdysis (E), the final mean $R_3$ R-value, and overall growth rate (ER).*

| Treatment (T) | Molt stage at T (after Skinner, 1962) | Mean $R_3$ value at T, (±SEM) | Sample size (n) | Mean number of days from T to ecdysis (E), (±SEM) | Mean final $R_3$ value (±SEM) | Mean overall growth rate (ER) T to E, (±SEM) |
|---|---|---|---|---|---|---|
| Control | $C_4$ | 0 | 40 | 96.8 (±5.4) | 22.50 (±0.5) | 18.1 (±1.9) |
| MA-CI | $C_4$ | 0 | 14 | 24.9 (±2.0) | 22.94 (±0.6) | 42.6 (±2.3) |
| MA | $C_4$ | 0 | 20 | 29.7 (±2.0) | 21.48 (±0.4) | 35.5 (±3.7) |
| ES | $C_4$ | 0 | 42 | 26.6 (±1.3) | 21.35 (±0.9) | 39.0 (±1.6) |
| Control | $D_0$ | 13.11 (±0.2) | 42 | 27.1 (±2.5) | 22.42 (±0.5) | 30.0 (±2.4) |
| MA-CI | $D_0$ | 12.80 (±0.5) | 15 | 25.1 (±1.1) | 23.20 (±0.4) | 28.6 (±2.4) |
| MA | $D_0$ | 13.32 (±0.5) | 15 | 28.0 (±1.5) | 23.00 (±0.4) | 28.0 (±2.4) |
| ES | $D_0$ | 12.52 (±0.4) | 24 | 18.7 (±1.5) | 22.18 (±0.4) | 30.9 (±1.7) |
| Control | $D_1$ | 20.30 (±0.7) | 27 | 11.7 (±2.0) | 24.00 (±0.4) | 20.9 (±5.6) |
| MA-CI | $D_1$ | 20.30 (±1.0) | 3 | 21.0 (±0.6) | 22.48 (±1.1) | 9.8 (±1.7) |
|  | $D_1$ | 23.16 (±1.3) | 5 | 4.6 (±1.7)* | 23.16 (±1.3) | 0 |
| MA | $D_1$ | 21.48 (±3.2) | 11 | 21.6 (±1.8) | 24.58 (±1.0) | 8.5 (±2.0) |
|  | $D_1$ | 22.08 (±2.2) | 2 | 5.5 (±1.5)* | 22.08 (±2.2) | 0 |
| ES | $D_1$ | 20.82 (±1.0) | 6 | 4.8 (±1.3) | 22.60 (±1.2) | 12.5 (±3.0) |

Means are given ± the standard error of the mean.

* MA-CI or MA limbs not regenerated.

TABLE III

*Autotomy-induced reductions in means (± standard error of means) of limb bud emergence time and/or reduction in the variance ($V = (y - \bar{y})^2/n - 1$) of bud emergence and intermolt cycle durations.*

| | | Mean number of days (±SEM) | | | Variances | | |
|---|---|---|---|---|---|---|---|
| Number of missing limbs (autotomized during stage $C_4$) | Sample size (n) | Autotomy to bud emergence | Bud emergence to ecdysis | Autotomy to ecdysis | Autotomy to bud emergence | Emergence to ecdysis | Autotomy to ecdysis |
| 1 | 41 | 43.5 (±3.6) | 65.8 (±5.0) | 110.4 (±5.8) | 518.7 | 760.3 | 1008.0 |
| 2 | 19 | 18.0 (±2.0) | 48.5 (±8.9) | 72.8 (±6.7) | 70.3** | 719.8† | 491.1 |
| 4 | 11 | 10.5 (±0.7) | 46.0 (±8.7) | 73.5 (±6.8) | 4.2! | 682.0†† | 510.8 |
| 3 + Cheliped | 16 | 7.8 (±0.8) | 40.6 (±5.0) | 51.0 (±3.9) | 10.3 | 327.8 | 203.1 |
| 8 (MA-CI) | 37 | 7.7 (±0.4) | 19.2 (±2.9) | 28.6 (±1.6) | 5.1 | 75.8 | 38.2 |
| 7 + Cheliped (MA) | 35 | 8.7 (±0.5) | 23.0 (±2.4) | 33.0 (±2.3) | 8.4 | 73.8 | 91.7 |
| 1* | 12 | 8.7 (±0.7) | 57.1 (±8.7) | 60.1 (±8.6) | 5.4 | 601.9 | 598.8 |

The pooled variance ratios were calculated to test the equality of variance and the variance ratio (F) was considered significant at $P < 0.05$.

Abbreviations are as in Table II.

* Autotomized following MA-induced ecdysis.

** F = 7.4 ($P < 0.01$).

! F = 16.7 ($P < 0.01$).

† F = 1.06 ($P > 0.05$).

†† F = 1.06 ($P > 0.05$).

Following the emergence of the limb bud papilla, a small limb bud begins to grow. This portion of limb regeneration is called basal growth (Bliss, 1956). In *Uca*, an $R_3$ bud will reach R-values of 10 to 13 during basal growth. Basal growth in control crabs is limited to stage $C_4$. The growth rate (ER) of the limb bud during this period is very slow (less than 18) and the small amount of growth that does occur may occur in discontinuous spurts.

In control crabs, rapid proecdysial growth begins at approximately 75% of the entire intermolt cycle. The ER of the limb bud may reach values of 30 to 40 (Table II). The limb bud grows and differentiates, and the muscles, chromatophores, and

TABLE IV

*The effects of multiple autotomy (seven walking legs plus cheliped = MA) and eyestalk removal (ES) performed separately (ES or MA) or together (ES plus MA) on mean intermolt cycle duration (in days).*

| | Mean initial $R_3$ value (±SEM) | | | Mean number of days (±SEM) | |
|---|---|---|---|---|---|
| | at ES | at MA | Sample size (n) | ES to E | MA to E |
| ES plus | 0 | — | 16 | 27.6 (±2.2) | — |
| MA | 0 | 3.28 (±0.7) | 8 | 33.3 (±1.5) | 24.0 (±1.3) |
| | 0 | 12.36 (±0.4) | 12 | 36.2 (±1.4) | 20.1 (±0.6) |
| | 0 | 19.75 (±0.7) | 10 | 22.0 (±1.8) | 6.1 (±1.2)* |
| ES or | 0 | 0 | 42 20 | 26.6 (±1.3) | 29.7 (±2.0) |
| MA | 2.49 (±0.2) | 2.56 (±0.5) | 14 16 | 33.4 (±1.9) | 29.4 (±1.7) |
| | 11.54 (±0.2) | 12.59 (±0.6) | 12 11 | 20.2 (±1.9) | 27.6 (±1.7) |
| | 20.82 (±1.0) | 20.23 (±0.7) | 4 11 | 7.5 (±1.6) | 19.7 (±1.8) |

* No regeneration of MA limbs.

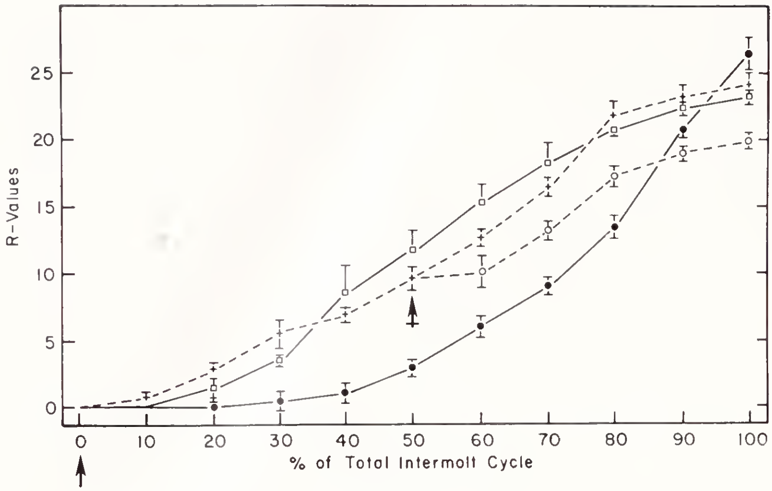


FIGURE 3. Comparison of the patterns of $R_3$ limb regeneration in controls (solid circles, solid line), animals missing seven walking legs plus cheliped (=MA, open squares, solid line) and eyestalkless crabs (crosses, dashed line). The arrow represents the time of MA and eyestalk ablation. The crossed arrow indicates the point at which eyestalkless crabs were forced to autotomize seven walking legs plus cheliped. The subsequent growth pattern is shown (open circles, dashed line). Each point represents the mean of at least six crabs, and the vertical lines represent standard errors of the means.

segmentation of the new limb become visible within the thin cuticle sac that covers the bud.

Regardless of the length of the intermolt cycle, the final R-values of the limb buds of control crabs are consistent (Table II). A long cycle does not result in a bigger limb bud nor does confinement to a short cycle limit the final size of the bud. A limb bud continues to grow until ecdysis. There is some "terminal plateau" (Bliss, 1956) during late proecdysis in control crabs (Fig. 3). The ER's of the limb bud are low prior to ecdysis (Table II).

At an R-value of 22 to 23, the control crabs shed the old exoskeleton. As the exoskeleton is discarded, the regenerated bud unfolds and expands. The only visible differences in a newly regenerated post-molt walking leg are its slightly smaller size and lighter color.

*Carapace and cheliped growth.* Following molt, the new carapace of control crabs increases in width by 2.4% (Table V). The average increase in the size of the cheliped of control crabs is 1.2%. The cheliped increases less than does carapace width. Therefore, there is a slight reduction of the cheliped/carapace ratio at each ecdysis in control crabs (Table VI). A cheliped from a control crab contains about 50% water and 7.6% protein (Table VII).

### *Growth patterns following autotomy*

*Regeneration of walking legs.* Multiple autotomy not only affects the duration of the induced proecdysial period, it also has an effect upon the pattern of growth of the regenerating limb buds (Fig. 3). The limb buds of MA and MA-CI animals emerge sooner after autotomy than do the limb buds of control animals (Table III), and all of the MA and MA-CI buds emerge simultaneously. The average rate of growth (ER) of $R_3$ limb buds from MA crabs autotomized during stage $C_4$ is sig-

TABLE V

*The effects of various treatments upon mean % changes in carapace and cheliped sizes and ratios.*

| Treatment | Sample size (n) | Mean carapace width (in mm)* (±SEM) |  | Mean % change (+ = increase and − = decrease) (±SEM) | Mean length of cheliped (in mm) (±SEM) |  | Mean % change (+ = increase and − = decrease) (±SEM) | Mean cheliped/carapace ratio (±SEM) |  |
|---|---|---|---|---|---|---|---|---|---|
|  |  | Prior to E (or to MA, or MA-CI) | After E |  | Prior to E (or to MA, or MA-CI) | After E |  | Prior to E (or to MA, or MA-CI) | After E |
| Controls | 30 | 15.53 (±0.2) | 15.91 (±0.2) | +2.4 (±0.2) | 21.47 (±0.5) | 22.12 (±0.6) | +1.2 (±0.4) | 1.38 (±0.02) | 1.38 (±0.03) |
| MA-CI | 13 | 15.43 (±0.6) | 15.45 (±0.4) | +0.12 (±0.8) | 20.60 (±1.5) | 20.80 (±1.4) | +1.3 (±0.7) | 1.31 (±0.08) | 1.36 (±0.07) |
| MA | 17 | 15.24 (±0.3) | 14.97 (±0.7) | −2.0 (±0.7) | 20.75 (±1.5) | 9.69 (±0.8) | −55.6 (±1.5) | 1.41 (±0.04) | 0.64 (±0.02) |
| 2nd Post-autotomy E | 16 | 16.19 (±0.4) | 16.33 (±0.4) | +1.1 (±0.3) | 11.27 (±0.6) | 14.44 (±0.8) | +28.0 (±3.4) | 0.69 (±0.02) | 0.88 (±0.04) |
| Claw only missing | 13 | 15.36 (±0.5) | 15.56 (±0.6) | +0.93 (±0.8) | 18.84 (±2.2) | 10.40 (±0.8) | −43.7 (±2.5) | 1.12 (±0.1) | 0.70 (±0.04) |
| 4 walking legs (CI) | 10 | 16.28 (±0.6) | 16.74 (±0.6) | +0.87 (±0.2) | 23.92 (±1.4) | 23.89 (±1.3) | −0.11 (±0.4) | 1.50 (±0.03) | 1.50 (±0.03) |
| 3 walking legs + cheliped | 14 | 14.88 (±0.4) | 14.91 (±0.4) | +0.58 (±0.5) | 21.02 (±0.2) | 10.98 (±0.7) | −45.6 (±4.1) | 1.38 (±0.1) | 0.71 (±0.02) |
| ES | 39 | 15.76 (±0.2) | 17.53 (±0.3) | +11.1 (±0.8) | 22.78 (±0.6) | 23.69 (±0.7) | +3.6 (±0.6) | 1.49 (±0.04) | 1.40 (±0.04) |
| ES (subsequent MA) | 12 | 15.90 (±0.4) | 17.01 (±0.4) | +7.4 (±1.4) | 22.52 (±1.4) | 5.82 (±0.3) | −73.3 (±2.6) | 1.31 (±0.06) | 0.34 (±0.02) |

Abbreviations are as in Table II.

* Measuring Error = 0.56%.

TABLE VI

*Changes in cheliped/carapace ratios (C/C) following three successive ecdyses.*

| | Mean cheliped/carapace ratios (±SEM) | | | | | |
|---|---|---|---|---|---|---|
| | Following ecdyses no: | | | | | |
| | 1 | | 2 | | 3 | |
| Limbs missing: | C/C ratio | Sample size (n) | C/C ratio | Saple size (n) | C/C ratio | Sample size (n) |
| 1 Walking leg | 1.43 (±0.02) | 43 | 1.35 (±0.03) | 23 | 1.31 (±0.04) | 7 |
| 8 Walking legs (MA-CI) | 1.41 (±0.04) | 14 | 1.36 (±0.03) | 3 | 1.34 (±0.07) | 3 |
| 1 Cheliped | 0.69 (±0.02) | 13 | 0.83 (±0.02) | 9 | 1.04 (±0.05) | 6 |
| 3 Legs + cheliped | 0.71 (±0.02) | 12 | 0.90 (±0.04) | 6 | 1.10 (±0.02) | 6 |
| 7 Legs + cheliped (MA) | 0.69 (±0.01) | 40 | 0.82 (±0.02) | 27 | 0.97 (±0.04) | 10 |

nificantly higher than the ER of $R_3$ limb buds from control crabs (Table II). However, if MA occurs during the early proecdysial period ($D_0$), the ER of the bud is no different from that of the controls (Table II).

The final R-values of $R_3$ limb buds from MA crabs are the same as the final $R_3$ values for the controls (Fig. 3 and Table II). Yet, the post-ecdysial size of newly regenerated limbs is considerably smaller than the size of control limbs (Table VII). A non-regenerated walking leg has an average of 2.6 mg of protein, and a regenerated $R_3$ has 1.0 mg of protein. However, the ratio of the total amount of protein/volume (= propus length$^3$) is the same in newly regenerated walking legs as in non-regenerated legs (Table VII).

*Carapace growth.* Following a multiple autotomy-induced ecdysis, the amount of growth (expressed as increase in carapace width) is significantly reduced when compared to controls (Table V).

Crabs that regenerate eight walking legs (MA-CI) increase only 0.12% in carapace width. Crabs that have a heavier regeneration load (*i.e.* seven walking legs plus the cheliped = MA) actually decrease in width by 2.0%. These crabs, however, increase in size following the second post-autotomy ecdysis and continue to get larger at each succeeding ecdysis. By the end of the sixth post-autotomy cycle, MA crabs are not significantly smaller than control crabs (Figs. 1a and 1b).

Regeneration of the large cheliped can, by itself, reduce the amount of post-autotomy growth: crabs regenerating a single walking leg and a cheliped show less increase in carapace width following ecdysis than do controls (Table V).

*Cheliped growth.* A regenerated cheliped is always very small. The cheliped/carapace ratio of newly regenerated chelipeds is about 0.70 (Table VI). Crabs are unable to regenerate a full-sized cheliped regardless of the size of the total regeneration load (Table V). A small cheliped, however, grows at each ecdysis (Table VI) and the ratio of protein to volume is not significantly less than the ratio for non-regenerated chelipeds (Table VII). Crabs that have lost eight walking legs but not the cheliped are able to maintain the growth of the cheliped at each ecdysis (Tables V and VI). These crabs do not appear to regenerate walking legs at the expense of the cheliped. The percent size increase of the cheliped of these crabs is the same as the percent increases of controls. The regeneration of eight walking legs is accomplished at the expense of carapace growth and not cheliped growth (Table V).

TABLE VII

*Mean (±SEM) water and protein content of chelipeds and walking legs following variously induced ecdyses.*

| Limbs: Type of induced molt | Sample size (n) | % $H_2O$ (of wet weight) (±SEM) | % Protein |  | Mean total amount of protein (mg) (±SEM) | Mean length of propus (cm) (±SEM) | Mean ratio of total amount of protein/cm$^3$ (= cube of propus length) (±SEM) |
|---|---|---|---|---|---|---|---|
|  |  |  | of wet weight (±SEM) | of dry weight (±SEM) |  |  |  |
| Chelipeds: |  |  |  |  |  |  |  |
| 1) Controls | 10 | 50.3 (±0.30) | 7.6 (±0.5) | 15.2 (±1.0) | 32.2 (±8.6) | 2.84 (±0.10) | 1.75 (±0.50) |
| 2) MA | 10 | 69.6 (±2.5) | 2.5 (±0.3) | 6.2 (±1.0) | 1.8 (±0.4) | 1.10 (±0.09) | 1.40 (±0.21) |
| 3) ~~ES~~ | 6 | 81.7 (±2.2) | 1.0 (±0.1) | 5.3 (±0.4) | 6.7 (±0.7) | 2.73 (±0.06) | 0.32 (±0.12) |
| 4) ~~ES~~ + MA | 3 | 80.9 (±4.6) | 2.0 (±0.5) | 10.9 (±2.9) | 0.34 (±0.1) | 0.60 (±0.04) | 1.47 (±0.39) |
| Walking legs: |  |  |  |  |  |  |  |
| 1) Controls | 27 | 54.2 (±0.6) | 8.8 (±1.2) | 12.3 (±4.7) | 2.6 (±1.0) | 0.89 (±0.20) | 5.97 (±1.2) |
| 2) MA | 11 | 68.5 (±4.5) | 6.0 (±1.0) | 14.5 (±4.4) | 1.0 (±0.4) | 0.60 (±0.06) | 5.15 (±2.2) |

Abbreviations are as in Table II.

A large, non-regenerated cheliped (C/C ratio = 1.66) contains an average of 32.2 mg of extractable protein (Table VII), whereas a regenerated cheliped (average C/C ratio = 0.81) has an average of 1.8 mg of protein. When a crab loses approximately 50 mg of protein (32.2 mg of cheliped protein and 18–20 mg of walking leg protein) through multiple autotomy, it regenerates only 8–9 mg of protein (approximately 1.8 mg of cheliped protein and 7–8 mg of walking leg protein).

### *Growth patterns following eyestalk removal*

*Regeneration of walking legs.* Figure 3 illustrates an averaged growth curve for regenerating $R_3$ limb buds from eyestalkless crabs (crosses, dashed line). Rapid proecdysial limb bud growth begins soon after eyestalk removal. The growth curve for this $R_3$ is parallel to, but ahead of, the curve for control crabs. The $R_3$ limb bud of an eyestalkless crab (like the limb bud of a MA crab) has an exaggerated period of no growth or terminal plateau at the end of the proecdysial period prior to ecdysis.

Growth of an $R_3$ from an eyestalkless crab can be inhibited during $C_4$ or $D_0$ by multiple autotomy (Fig. 3, crossed arrow). The inhibition lasts until the newly autotomized papillae emerge, then growth of all limb buds continues at ER's comparable to other eyestalkless crabs. These crabs enter terminal plateau at $R_3$ values significantly lower than the final $R_3$ values of intact controls and eyestalkless (but otherwise untreated) crabs (Fig. 3).

Frequently, when eyestalks are removed at the same time as autotomy, the limb bud papilla will not emerge and the crab will molt without any regeneration. In most of the experiments reported here, the $R_3$ limb papillae were allowed to emerge prior to eyestalk removal. In about 25% of the experimental crabs, eyestalk removal did not cause limb bud growth or ecdysis. These unresponsive crabs remained alive for considerable lengths of time, then died. They generally died prior to the ecdysis of the other eyestalkless crabs.

*Carapace growth.* Eyestalk removal results in an 11.1% increase in carapace width (Table V). This increase is reduced to 7.4% if eyestalk removal is followed by multiple autotomy of seven walking legs plus the cheliped (Table V).

*Cheliped growth.* Chelipeds from recently molted, eyestalkless crabs have the same linear dimensions as do the chelipeds from control crabs (Table VII). However, the chelipeds from eyestalkless crabs contain relatively less protein and more water than do the chelipeds from controls, and the ratio of the amount of protein to cheliped volume is significantly less than controls (Table VII). When the cheliped and several walking legs are autotomized from an eyestalkless crab, the regenerated cheliped is even smaller (C/C = 0.34) and contains much less protein. The protein to volume ratios in these claws, however, are similar to the controls (Table VII).

## DISCUSSION

When male specimens of *Uca pugilator* are kept in the laboratory in constant environmental conditions (23°C, 12 hours light/day, private boxes, and oatmeal once per week) these crabs will molt and grow. The intermolt cycles of these animals are extremely variable. The crabs molt independently of one another and intermolt cycle durations vary dramatically from crab to crab and from cycle to cycle (in intact control crabs lacking one walking leg). If *Uca* are held in the lab in constant conditions for several months, there is a reduction in the mean of the molt cycle due to a reduction in the number of extremely long intermolt periods. The mean of these later intermolt cycles drops to about 70 days, but the unpredictable and variable molting patterns for individual crabs remain unchanged.

The crabs used in these experiments were collected from populations of crabs in Florida. The climate in Florida is probably less of a limiting factor to food getting and reproduction than in more temperate regions. Environmental clues serve to synchronize feeding, reproductive, and molting activities of some populations. Since natural populations of *Uca* molt in burrows (away from other members of the population) and females copulate in a hardened, intermolt stage (rather than being restricted to the shorter and softer post-molt stage) there would be no obvious survival or reproductive advantage for the members of the population to molt in synchrony (as do some of the aquatic crabs and shrimps). It is not surprising, therefore, that external clues seem to be less important in controlling intermolt cycles in Florida populations of *Uca* than has been reported for other crustaceans (Bliss and Boyer, 1964; Weis, 1976). Crane (1975) has suggested that much of the ritualistic intermale combat and courting behavior observed in populations of *Uca* in the field, serves to synchronize certain group activities. The vast differences in intermolt cycle durations reported here may be due, in part, to the fact that these experimental crabs were held in individual boxes. Crabs held apart are deprived of any social synchronization.

Although individual crabs held in constant conditions continue to molt independently of one another, they can be induced (by multiple autotomy and eyestalk removal) to enter proecdysis and molt in concert. However, the two induced proecdyses are very different: while MA and MA-CI seem to reset a highly controlled and biphasic program, eyestalk removal appears to simply remove endogenous inhibitory mechanisms (that in control animals are withheld only during late proecdysis).

The response to multiple autotomy in *Uca* is divided into two distinct phases. The first phase consists of a physiological resetting. In *Uca*, the "reset event" is (1) independent of the eyestalks; (2) inhibitory to proecdysis; and (3) the initial response to autotomy. Skinner and Graham (1972) suggested that multiple autotomy in crabs resets the entire intermolt cycle. In *Uca*, this does not seem to be the case. It appears that the reset effect of multiple autotomy is independent of the effect of multiple autotomy upon the duration of the subsequent proecdysial period. The number of days from MA (or MA-CI) to ecdysis is consistent regardless of whether MA occurs during $C_4$ or early D (Table II). But when MA occurs in eyestalkless animals, only the reset effect is observed. MA seems to have no effect on the proecdysial program when eyestalks are missing.

In *Uca*, autotomy-induced resetting allows for the emergence and early growth of autotomized limb buds. Adiyodi (1972) has shown that the earliest phase of regeneration (limb bud emergence and basal growth) in the crab *Paratelphusa* is characterized by extensive mitotic activity and is different from the actual proecdysial growth phase which is characterized by increased cell size rather than number. Limb bud emergence and basal growth are independent of proecdysis and are inhibited if autotomy occurs during the later stages of D (Bliss, 1956; Passano and Jyssum, 1963; Hopkins, *et al.*, 1979). Thus, when a limb is lost, it is necessary to establish the internal physiological conditions that will allow for the mitotic events of blastema organization and limb bud papillae emergence. If the function of the reset event is to allow blastema organization and early bud growth, then the reset event is not limited to multiple autotomy. The loss of a second walking leg during $C_4$ has a profound effect on the growth of the previously autotomized limb bud. The basal growth of the first limb bud is inhibited until the emergence of the second limb papilla. Both of these limb buds will then proceed through basal growth simultaneously. The duration from autotomy until emergence of the second papilla is significantly shorter than the time for emergence of the first limb. The simultaneous loss of two limbs during $C_4$ hastens the emergence of both limb papillae (Table III). Autotomy of two limbs has a reset effect that is less than the effect of autotomy of four limbs or of MA.

The resetting event that allows for emergence of the blastema also seems to have an effect on the time that it takes the animal to reach proecdysis. There is a decrease in the number of days from autotomy to ecdysis with increasing numbers of limbs removed. Thus, there is a cumulative effect of limb loss upon the onset of proecdysial program in *Uca*. Each limb adds to the overall effect. Fingerman and Fingerman (1974) have reported in female *Uca pugilator*, an increase in molting rate (expressed as percent ecdysis/time) with increased numbers of limbs removed. Weis (1977b) reported that multiple autotomy during early proecdysis ($R_1$ value of 10) accelerated the growth of the original $R_1$ and hastened the onset of ecdysis in *Uca*. She also reported that autotomy of five or more limbs had a greater acceleratory effect than autotomy of two limbs. In describing the effects of limb loss on molt cycle in the cockroach, *Blattella*, Kunkel (1977) suggested that there is an independent signal from each regenerating limb with an average delay message programmed for each autotomized limb in the hemiganglion serving that limb. A similar model may be applicable to *Uca*, with each limb having an individual message and the final effect being the sum of those messages.

The extremely large cheliped of *Uca* has a greater resetting effect than does a single walking leg. Emergence of limb papillae in response to autotomy of four walking legs lags behind limb papillae emergence in response to loss of three walking legs and the cheliped. Also, autotomy of eight walking legs is less effective in causing a reset event in late proecdysis than is autotomy of seven walking legs plus the cheliped. These results differ from those reported for the tropical land crab, *Gecarcinus lateralis* (Skinner and Graham, 1972). In *Gecarcinus*, loss of a cheliped was no more effective than loss of a walking leg in inducing proecdysis. The large cheliped of *Uca*, however, is relatively much larger than either of the chelipeds of *Gecarcinus* and may play a more important role in the social and reproductive behavior of *Uca* than do the two chelipeds of *Gecarcinus*. Therefore, there may be a greater advantage to *Uca* to preferentially regenerate the cheliped.

The second phase of an autotomy-induced cycle is the actual growth phase of "proecdysial program." This program is (1) normally under the control of the eyestalks; and (2) disrupted by the reset event. The proecdysial duration of crabs missing eight walking legs (MA-CI) is the same as that of crabs missing their eyestalks and of control crabs (25 to 27 days). This is a significantly shorter duration than the duration from MA to ecdysis in eyed crabs (33 days). If 25–27 days represents the shortest proecdysial duration, then loss of the cheliped must exert some inhibitory control over the onset or duration of the proecdysial program. This inhibitory control is mediated through the eyestalks because MA of eyestalkless crabs resets but does not affect the proecdysial program. Likewise, MA during $D_1$ in intact crabs resets but does not affect the subsequent proecdysial program. Thus, in crabs with minimal (or no) eyestalk controls, MA can only initiate the reset event and has no control over the proecdysial program.

Eyestalk removal in *Uca* does not always result in regeneration and ecdysis. Up to 25% of destalked *Uca* do not respond to eyestalk removal. Charmantier-Daures (1976) reported that during stage $C_4$, eyestalk removal in the crab, *Pachygrapsus*, induced regeneration in only 50% of the crabs. Perhaps, these unresponsive crabs are physiologically inadequate to initiate the processes that lead to ecdysis. Unlike the crab *Gecarcinus*, eyestalkless *Uca* do not always die at or before molt. About 20% of eyestalkless *Uca* live through two ecdyses and the length of the second intermolt is virtually the same as the first intermolt duration.

It has been proposed that the effects which follow autotomy in crustaceans are due to the severance of a critical number of leg nerves (Skinner and Graham, 1972; Bittner and Kopanda, 1973). This "severed nerve hypothesis" would not, however, account for the fact that in *Uca* autotomy of the cheliped has a greater effect than autotomy of a single leg. Nor could it account for the fact that the duration of the second post-autotomy intermolt cycle is significantly shorter than the comparable intermolt cycle of the controls. (Charmantier-Daures, 1976, observed a similar effect in the crab, *Pachygrapsus*.) These facts suggest that a message with qualitative and quantitative information about the limb is conveyed to the CNS and the message is not merely an on/off signal as suggested by the severed nerve hypothesis. Newly regenerated limbs are smaller after molt than non-regenerated limbs (see below) and slight injuries may occur to the new limbs during the extremely difficult task of getting out of an old exoskeleton with a minimum number of limbs and efficiency. Minor injuries and/or small limb size may alter or modify the messages sent back to the CNS by the intact limbs. The "program" may also respond to sensory input: smaller, newly regenerated limbs may not have as many sensory structures as non-regenerated limbs.

The effects of MA (and MA-CI) are evident in the growth rates of the regenerating limb buds. MA during intermolt speeds the ER's of the resulting limb buds. During mid-proecdysis, the rates of growth are unaffected and in late proecdysis the overall rates of limb bud growth are slowed. The final size of the regenerated limb bud does not appear to be affected by speeding or slowing the growth rates. The final size of the limb buds are the same for limb buds that have regenerated slowly and buds that have regenerated quickly.

In *Uca*, ecdysis does not always result in an increase in carapace size (see also Guyselman, 1953; Weis, 1976). Ecdysis may take place solely as a means of regenerating missing limbs, and sometimes regeneration may take place at the expense of general body growth. Under the holding conditions described here, crabs that regenerate more than four legs possess a new carapace that is no larger and sometimes smaller than the one shed. There is a relationship between regeneration load and degree of growth (or no growth) observed in the post-molt carapace. Fingerman and Fingerman (1974) reported that intact female *Uca* regenerating eight walking legs showed less growth than intact crabs missing only one limb, but they did not report any loss of carapace size. The new exoskeleton of a post-ecdysial crab is initially expanded with water taken up and stored during proecdysis (Bliss and Boyer, 1964) and during post-molt the fluid is replaced with protein (Skinner, 1966). Perhaps the volume of water taken up during proecdysis is the same whether the crab is or is not regenerating limbs. During post-molt, then, an MA crab must use that volume of water to expand not only the new exoskeleton carapace but also the newly regenerated cheliped and all of the new walking legs. The reduction in carapace size (or lack of increase in size) might, therefore, be due to insufficient water uptake during proecdysis.

The failure to increase in size at ecdysis is not due to the truncated proecdysis. Eyestalkless crabs have the briefest proecdysial duration, yet eyestalkless crabs have the largest post-ecdysial increase in carapace size. MA reduces the post-molt increase in size of eyestalkless crabs. If the increase in carapace size in eyestalkless animals is due to increased water uptake, then MA may block the increase in carapace size in much the same way that it may block the increase in intact crabs.

Intact control crabs do not always have a terminal plateau at the end of the proecdysial period. Terminal plateau (a period of no growth preceding ecdysis) occurs consistently in eyestalkless and, to a lesser extent, in MA and MA-CI crabs. Crabs missing seven or eight limbs show some terminal plateau, but less terminal plateau is evident in crabs missing fewer limbs. Perhaps regeneration becomes uncoupled from other proecdysial events in those crabs that have an exaggerated terminal plateau. The fact that eyestalkless crabs (with subsequent MA) have a terminal plateau at R-values that are significantly lower than in eyestalkless crabs suggests that terminal plateau is not due to limb buds having reached maximal size, but rather is due to physiological conditions at the end of proecdysis that are inhibitory to further growth of the limb buds. At ecdysis, eyestalkless crabs have buds that are the same size as the limb buds of intact crabs at ecdysis. The fact that these buds are no smaller than other buds is unexpected in light of the extreme differences found in size and protein content of the post-ecdysial limbs.

It has been reported in other crabs that post-molt regenerated limbs are smaller than post-molt non-regenerated limbs (Skinner and Graham, 1972; Charmantier-Daures, 1976). This is also true in *Uca*. Fingerman and Fingerman (1974) and Weis (1976) also reported that post-molt walking legs were smaller in MA *Uca*. Newly regenerated legs are 32% smaller than non-regenerated legs and contain 62% less protein. In *Uca* a regenerated cheliped is much smaller. Newly regenerated chelipeds increase in size with each succeeding ecdysis. The chelipeds increase 28% at the second post-autotomy ecdysis and continue to increase at each ecdysis. Due to the high mortality rate for MA crabs, it was never observed whether the regenerated chelipeds ever regain their former dimensions.

Under the holding conditions described above, *Uca* is capable of *de novo* synthesis of only 9 mg of protein (regardless of how many limbs were lost through autotomy). This amount of protein is much less than the amount the crab *Gecarcinus* is capable of regenerating (Skinner and Graham, 1972). However, this difference may be due to the fact that *Gecarcinus* is a considerably larger crab.

Skinner (1966) reported that the amount of muscle per cheliped in *Gecarcinus* was lowest during the first few days after ecdysis and the maximal growth of the chelipeds (in terms of incorporation of $^{14}$C-leucine into protein) occurred during post-molt. The post-molt size of an unregenerated cheliped from an eyestalkless *Uca* has the same linear dimensions as the unregenerated cheliped from an eyed control crab. However, the ratio of protein to volume of the cheliped from the eyestalkless crab is greatly reduced. These chelipeds from eyestalkless crabs grow over 5% in linear dimensions following ecdysis but contain much less protein. This is probably due to the fact that these eyestalkless crabs have little or no post-molt, but rather pass very quickly from ecdysis into a new proecdysial period. Thus, eyestalkless crabs have less "down time" in which muscle protein can be synthesized to replace muscle protein autolysed during proecdysis. On the other hand, eyestalkless crabs that are subsequently autotomized (including the cheliped) are sufficiently inhibited by the resetting action of autotomy that they can regenerate the cheliped. The period of regeneration is so short, however, that the linear dimensions of the newly regenerated cheliped are only half the dimensions of chelipeds regenerated by intact crabs. Perhaps the physiological conditions of proecdysis are inhibitory to protein synthesis, or the autolysis of muscle that occurs during proecdysis is so extensive that it somehow overrides most synthetic efforts.

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# FINE STRUCTURE OF A SCYPHOZOAN PLANULA, *CASSIOPEIA XAMACHANA*

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## ABSTRACT

Pre-metamorphic planulae of the scyphozoan *Cassiopeia xamachana* contain four cell types. The ectoderm consists of supportive cells and differentiating nematoblasts and nematocytes, while the endoderm consists of supportive cells and interstitial cells. Neural elements and glandular cells are absent in these planulae. Morphological similarities and differences that exist among hydrozoan, scyphozoan, and anthozoan planulae are discussed.

## INTRODUCTION

Most cnidarians have a planula stage at some time in their life cycle. Planulae are cylindrical and are composed of an ectoderm and an endoderm separated by a thin mesoglea. In recent years several ultrastructural studies have described the morphology of hydrozoan and anthozoan planulae (Lyons, 1973a, b; Vandermeulen, 1974; Chia and Crawford, 1977; Martin and Thomas, 1977, 1980; Chia and Koss, 1979). The ultrastructural morphology of scyphozoan planulae has been largely ignored. Otto (1978) examined the morphological and ultrastructural changes which took place during settlement of scyphozoan planulae of *Haliclystus salpinx*. The planulae of this Stauromedusae are atypical in that they lack cilia, do not swim, and usually contain a constant number of endodermal cells. Since there has been no comprehensive fine-structural study to date describing a more typical scyphozoan planula, we examined the planulae of *Cassiopeia xamachana*. It is hoped that such a study might reveal possible morphological similarities and differences among hydrozoan, scyphozoan, and anthozoan planulae.

## MATERIALS AND METHODS

Adult *Cassiopeia* were collected in December, 1980 at La Paguera, Puerto Rico. Gonads and gastric filaments were removed from the adults, placed in finger bowls of filtered sea water, and macerated with a pipette. Young planulae were soon observed swimming in these containers. Four days after collection of planulae, swimming planulae were fixed for 2½ hours in 2.5% glutaraldehyde in 0.2 *M* phosphate buffer (Dunlap, 1966; Cloney and Florey, 1968). They were post-fixed for 2 hours in 2% osmium tetroxide, pH 7.2, in 1.25% sodium bicarbonate (Wood and Luft, 1965). Specimens for transmission electron microscopy were dehydrated in an ethanol series, infiltrated with propylene oxide, and embedded in Epon (Luft, 1961). Blocks were sectioned on a Porter Blum MT-2B ultramicrotome, placed on 150-mesh copper grids, and stained in 5% uranyl acetate in methanol followed by lead hydroxide. Grids were examined with a Phillips EM 201 transmission electron

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microscope. Planulae fixed for scanning electron microscopy were dehydrated through a graded series of amyl acetates, critical point dried, mounted on stubs and shadowed with carbon followed by gold. The specimens were examined with a Cambridge Stereoscan 150 SEM.

For histochemical studies and the detection of glandular cells, thick plastic serial sections, 1–3 μm thick, were mounted on glass slides. The Epon was removed according to the method of Lane and Europa (1965) and the sections were stained by the periodic acid-Schiff (PAS) procedure (Lillie, 1954).

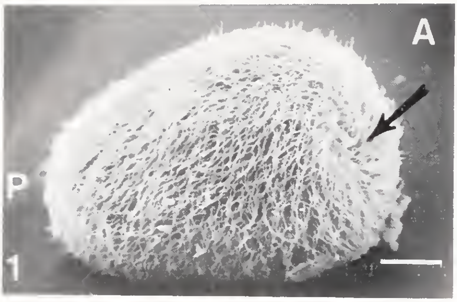
## RESULTS

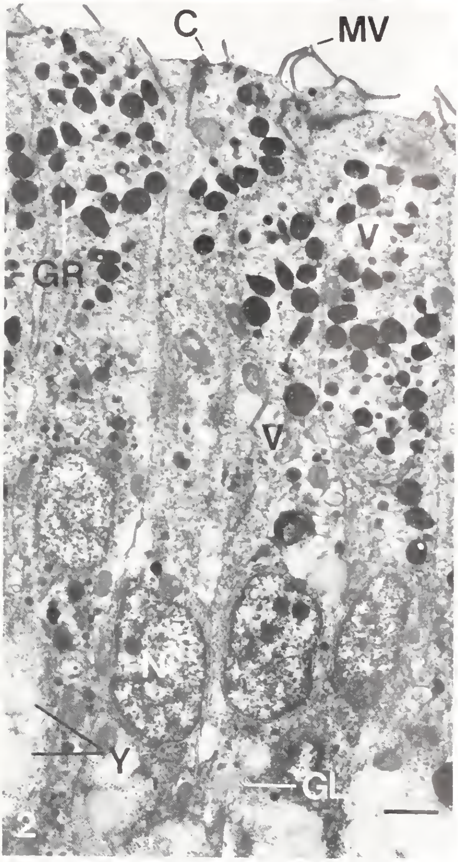
The pre-metamorphic planula of *Cassiopeia* ranges from 120 μm to 220 μm in length and from 85 μm to 100 μm in width in its mid-region. It is uniformly ciliated and swims with the enlarged anterior end forward. Just prior to metamorphosis, an indentation is found at the anterior end (Fig. 1). The majority of planulae observed settle on the bottoms of glass dishes and undergo metamorphosis within 4–5 days after collection. In some cases, planulae undergo metamorphosis without prior attachment to glass.

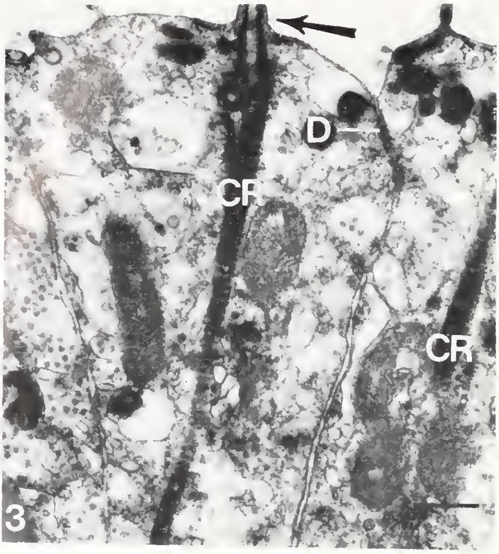
Fine-structural examination of pre-metamorphic planulae reveals only 4 cell types: 2 in the ectoderm and 2 in the endoderm. The ectoderm consists of supportive cells and differentiating nematoblasts and nematocytes. Supportive cells are columnar in shape and extend from the free surface of the planula to the mesoglea (Fig. 2). Each supportive cell bears microvilli and a single cilium at its apical surface (Figs. 2 and 3). The cilium is of the 9 + 2 microtubular arrangement and extends from the apical surface without a concavity. It consists of a basal plate located above a basal body and an accessory basal body (Fig. 4). The basal body gives rise to a striated ciliary rootlet with a periodic banding pattern of about 300 Å. The rootlet extends deep into the cytoplasm of the cell and terminates just above the nucleus. Attached to the accessory basal body is a plaque-like structure that parallels the ciliary rootlet (Fig. 4). Microfilaments of a terminal web are found directly beneath the apical surfaces of the cells (Fig. 5) and terminate at the lateral cell boundaries on either side. Septate desmosomes are present between these supportive cells in their apical regions (Fig. 6). Numerous electron-dense, membrane-bounded granules fill the apical regions of the cells (Fig. 2). Vacuoles are also present. The nucleus of each cell is centrally located and contains a nucleolus and condensed chromatin. A few Golgi complexes are located in close association with the nucleus. Mitochondria, polysomes, and endoplasmic reticulum are scattered throughout the cytoplasm.

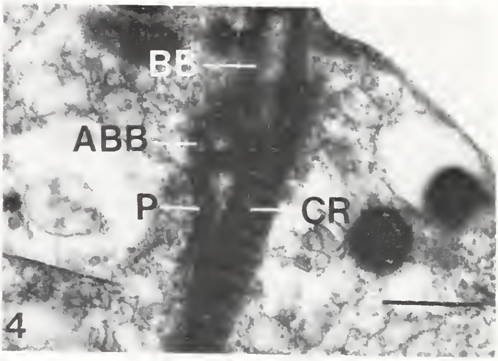
Basally, foot processes of the supportive cells insert on the mesoglea (Fig. 7). PAS-positive granules and glycogen particles are abundant in the basal regions of these cells. Specialized junctional complexes resembling desmosomes and hemidesmosomes are located between the foot processes of adjacent supportive cells and between the foot processes and the mesoglea (Fig. 8). Microfilaments are seen radiating out from dense regions located along the inner borders of the junctional membranes (Fig. 9). The two membranes are separated by a space of 150–200 Å.

Fully differentiated nematocytes are abundant at the ectodermal surfaces of planulae (Fig. 10). They are especially numerous in the anterior indentation region. The cells are embedded within the supportive cells and do not extend to the mesoglea. The nematocyst is large and occupies the upper two-thirds of the cell. A modified cilium gives rise to the cnidocil which is located to the side of the nematocyst. The capsule of the nematocyst consists of an outer electron-dense layer









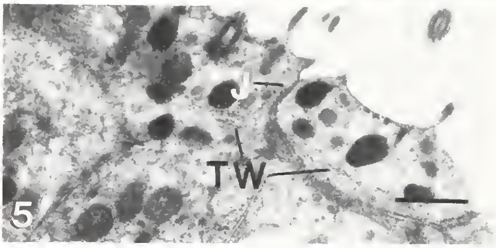


FIGURE 1. Scanning electron micrograph of a planula of *Cassiopeia*. The planula has a distinct anterior end and posterior end and is uniformly ciliated. Just prior to attachment and metamorphosis, an indentation is found in the anterior end (arrow). A = anterior; P = posterior. Bar = 20 μm.

FIGURE 2. Transverse section of the apical regions of ectodermal supportive cells. These supportive cells possess a single cilium, numerous microvilli, and electron-dense granules. The nucleus of the cell is centrally located and contains a prominent nucleolus. PAS-positive granules and glycogen particles are located more basally in these cells. C = cilium; GL = glycogen particles; GR = granules; MV = microvilli; N = nucleus of supportive cell; V = vacuoles; Y = PAS-positive granules. Bar = 2 μm.

FIGURE 3. Cilium of ectodermal supportive cell. Each cilium projects directly from the apical membrane of the cell without a concavity (arrow). The ciliary rootlet extends deep into the cytoplasm of the cell. CR = ciliary rootlet; D = desmosome. Bar = 1 μm.

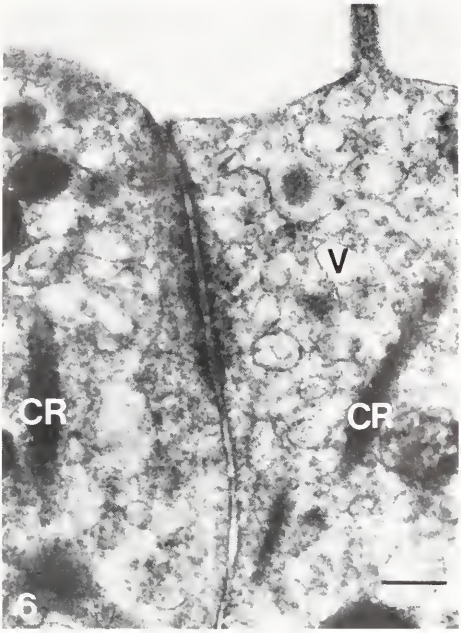
FIGURE 4. Basal body (BB), accessory basal body (ABB), ciliary rootlet (CR), and plaque-like structure (P) of a supportive cell. Bar = 1 μm.

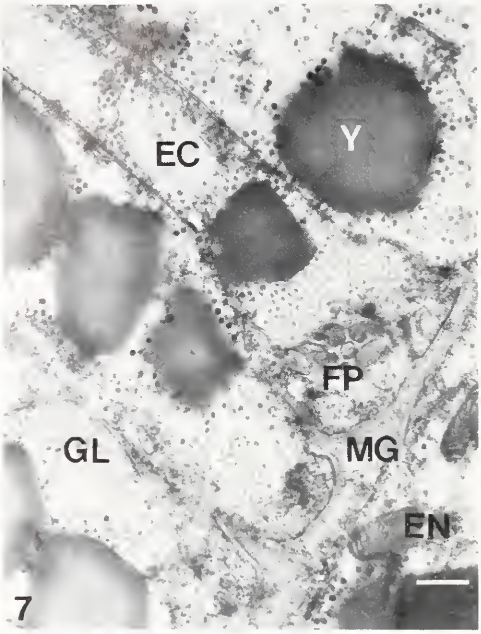
FIGURE 5. Terminal web beneath the apical membrane of a supportive cell. The microfilaments of the terminal web insert at the cell junctions. J = junction between cells; TW = terminal web. Bar = 2 μm.

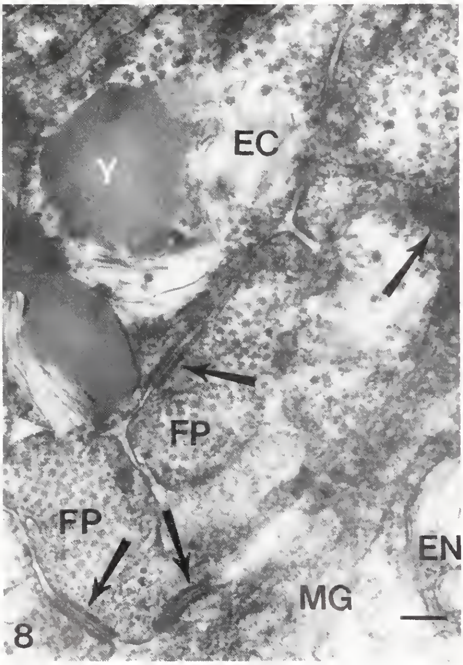
TABLE I

*Comparison of planulae.*

| Class | Anthozoa | | | | Hydrozoa | | Scyphozoa | |
|---|---|---|---|---|---|---|---|---|
| Species | *Anthopleura elegantissima* | *Ptilosarcus gurneyi* | *Pocillopora damicornis* | *Balanophyllia regia* | *Pennaria tiarella* | *Mitrocomella polydiademata* | *Haliclystus salpinx* | *Cassiopeia xamachana* |
| References | Chia and Koss (1979) | Chia and Crawford (1977) | Vandermeulen (1974) | Lyons (1973) | Martin and Thomas (1977); Martin (1980) | Unpublished observations | Otto (1978) | Unpublished observations |
| Age (post-fertilization in days) | 21 | 18 | 6 (?) | (?) | 3–6 | 3–5 | 5 | 3–6 |
| Size ($\mu$m) length × width | 200 × 150 | 1,000 × 300 | 1,000 × 500 | (?) | 650 × 80 | 200 × 70 | 100 × 20 | 120 × 85 |
| Types of cells in ectoderm | 9 | 7 | 9 | 10 | 7 | 7 | 3 | 2 |
| Types of cells in endoderm | 6 | 2 | 1 (?) | 1 (?) | 2 | 2 | 1(16 cells) | 2 |
| Uniformly ciliated | Yes | Yes | Yes | Yes (flagellated) | Yes | Yes | No | Yes |







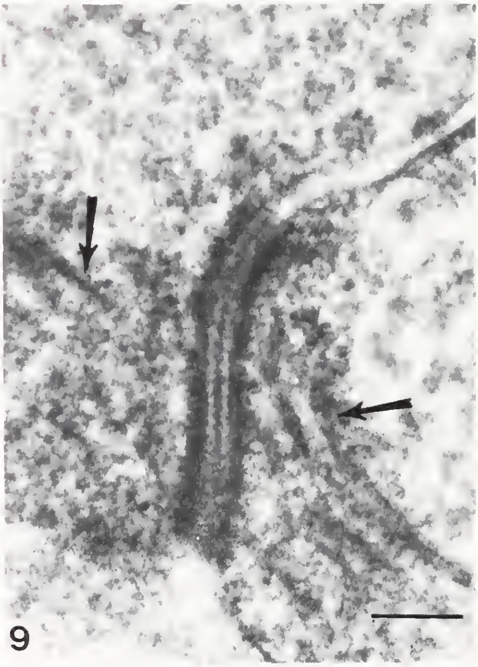


FIGURE 6. Septate desmosome between 2 supportive cells. CR = ciliary rootlet; V = vacuole. Bar = 0.5 μm.

FIGURE 7. Foot processes of the ectodermal supportive cells. These processes insert on the mesoglea and contain numerous PAS-positive granules and glycogen particles. EC = ectoderm; EN = endoderm; FP = foot process of supportive cell; GL = glycogen particles; MG = mesoglea; Y = PAS-positive granules. Bar = 1 μm.

and an inner electron-lucent layer. The thread of the nematocyst bears arms and spines and is coiled around a heavily barbed shaft. The nucleus of the cell is small and basally located. A well-developed Golgi apparatus is also present in the basal region of the cell. Endoplasmic reticulum is scarce.

Developing nematoblasts are located at the base of the epidermis among the foot processes of the supportive cells (Fig. 11). They do not make contact with the free surface of the ectoderm. The cytoplasm of these developing nematoblasts contains endoplasmic reticulum, a Golgi complex, and a developing nematocyst.

The endoderm is composed of a single layer of columnar-shaped cells very similar in structure to the supportive cells of the ectoderm (Fig. 12). These cells insert on the mesoglea *via* their basal ends. PAS-positive granules, vacuoles, glycogen particles, and electron-dense granules are abundant in these basal regions. Microfilaments are not detected in these cells. Apically the cells bear a single cilium surrounded by a collar of microvilli which projects into a forming gastrovascular cavity (Figs. 12 and 13). The nucleus is centrally located and often contains a nucleolus. Mitochondria, endoplasmic reticulum, and polysomes are found throughout the cytoplasm.

Clusters of interstitial cells are scattered among these supportive cells of the endoderm. The nucleus of each interstitial cell is round and contains a prominent nucleolus. Numerous free ribosomes are present in a homogeneous cytoplasm. Other organelles are sparse or poorly developed.

A thin mesoglea separates the ectoderm from the endoderm. The mesoglea consists of a meshwork of fibers which are oriented in all directions. These fibers are embedded within a PAS-positive, amorphous ground substance.

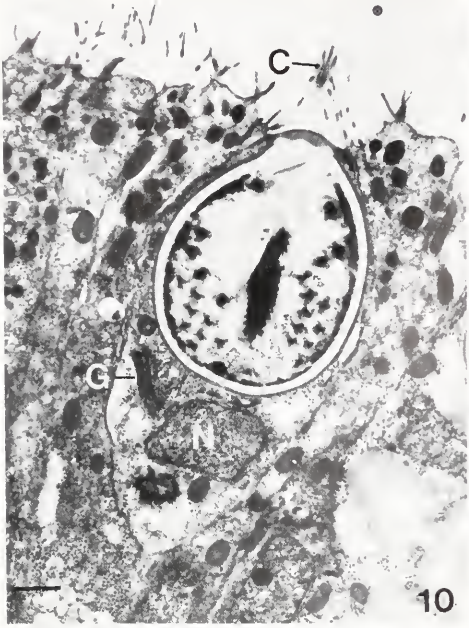
Examination of thick plastic serial sections and comparable thin sections repeatedly demonstrate the absence of both nerve cells and glandular cells in planulae of *Cassiopeia*. The negative PAS staining reaction also verifies the absence of glandular cells.

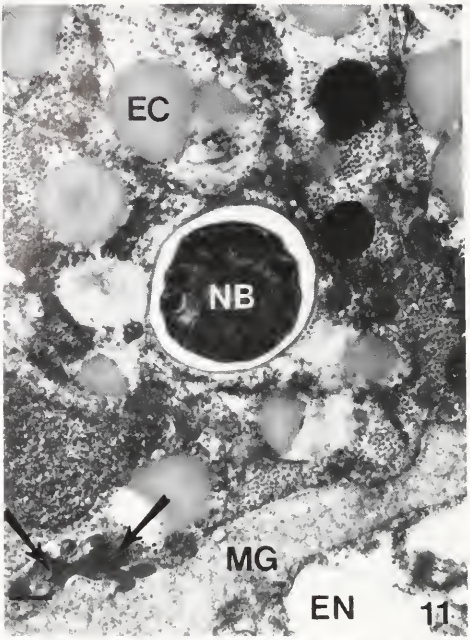
## Discussion

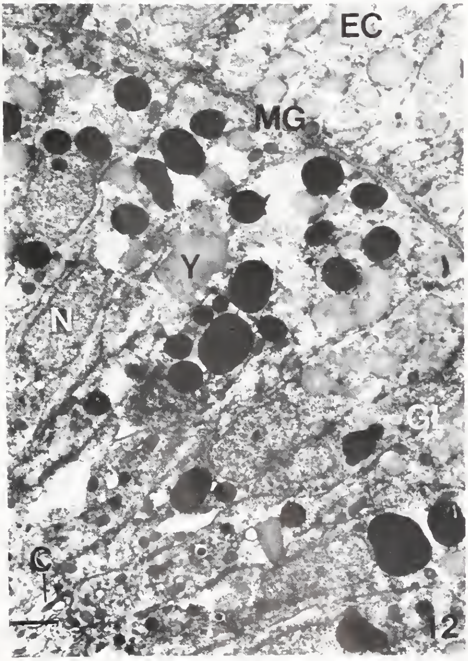
Results from this study and that of Otto (1978) indicate that planulae of scyphozoans are smaller in size and are morphologically simple when compared to planulae of hydrozoans and anthozoans (Table I). Planulae of *Cassiopeia* and *Haliclystus* are composed of only 4 cell types, whereas the hydrozoan planulae thus far examined contain 9 cell types, and the anthozoan planulae possess anywhere from 9 to 15 cell types. In *Cassiopeia* the planular ectoderm consists of 1 type of supportive cell and 1 type of nematocyte, while the endoderm contains interstitial cells and 1 kind of supportive cell. In *Haliclystus* 3 types of cells are present in the ectoderm (1 form of supportive cell, 1 form of nematocyte, and interstitial cells), and only 1 type of supportive cell comprises the endoderm. Otto (1978) reported microfilaments at the base of the supportive cells in both ectoderm and endoderm of planulae of *Haliclystus*. In planulae of *Cassiopeia*, however, microfilaments were found only

FIGURE 8. Specialized junctional complexes (arrows) between the foot processes of adjacent supportive cells and between the foot processes and the mesoglea. EC = ectoderm; EN = endoderm; FP = foot process of supportive cell; MG = mesoglea; Y = PAS-positive granules. Bar = 1 μm.

FIGURE 9. Specialized junctional complex between 2 foot processes of the supportive cells. These junctions are very similar to desmosomes in that the unit membranes appear thickened due to the presence of a dense amorphous layer closely applied to their cytoplasmic surfaces. Microfilaments (arrows) radiate out from this amorphous substance. A slender intermediate dense line is seen in the middle of the intercellular space between the 2 halves of the junction. Bar = 0.5 μm.







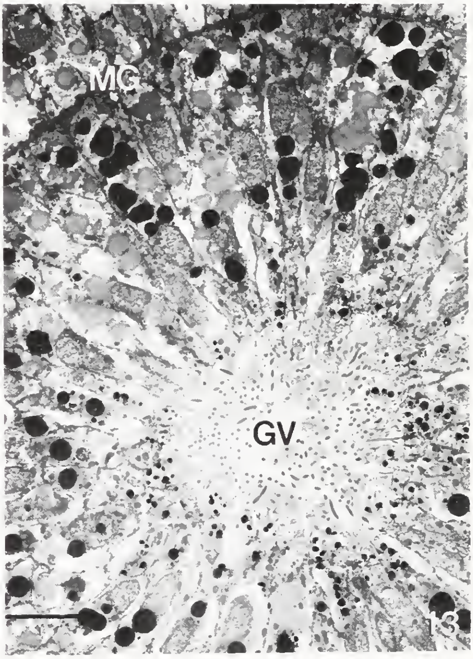


FIGURE 10. Nematocyte at the ectodermal surface of the planula. The nematocyte contains a large nematocyst and a basally located nucleus. A well-developed Golgi body is usually found in a supranuclear position. C = cilium; G = Golgi body; N = nucleus. Bar = 2 μm.

FIGURE 11. Developing nematoblast located at the base of the ectoderm among the foot processes of the supportive cells. Specialized junctions between the foot processes of the supportive cells can be seen (arrows). EC = ectoderm; EN = endoderm; MG = mesoglea; NB = nematoblast. Bar = 1 μm.

in the apical cytoplasm and the foot processes of the ectodermal supportive cells. Both the planulae of *Cassiopeia* and *Haliclystus* lack glandular cells and neural elements which generally are present in hydrozoan and anthozoan planulae (Lyons, 1973a, b; Vandermeulen, 1974; Chia and Crawford, 1977; Martin and Thomas, 1977, 1980; Chia and Koss, 1979).

Comparisons of the ultrastructural morphology of planulae from the 3 classes of cnidarians may add important insights into the phylogenetic classification of the cnidarians. Planulae of *Pennaria* (Martin and Thomas, 1977, 1980) and *Mitrocomella* (Martin *et al.*, unpublished observations) have 7 types of cells in the ectoderm and 2 kinds of cells in the endoderm. These hydrozoans are similar to the scyphozoan planulae in that in both classes the supportive cells of the ectoderm and the endoderm are arranged in a simple columnar epithelium with basal foot processes that insert on a thin mesoglea. Also, in both classes the planulae contain only 1 type of nematocyte. The 2 classes differ in that the hydrozoans contain neurosensory cells, ganglionic cells, and 2 types of glandular cells in the ectoderm. Anthozoan planulae, when compared to planulae of hydrozoans and scyphozoans, tend to show an increase in the types of glandular cells, the types of supportive cells, the types of nematocytes, and the complexity of the nervous system. The ectoderm of anthozoan planulae may be simple columnar, pseudostratified, or stratified depending upon the species examined (Vandermeulen, 1974; Chia and Crawford, 1977; Lyons, 1973a). Planulae of *Ptilosarcus* have 2 types of supportive cells and 3 types of glandular cells in the ectoderm (Chia and Crawford, 1977). In *Pocillopora* and *Balanophyllia* 3 types of nematocytes and 4 kinds of secretory cells are found in the ectoderm of the planulae (Vandermeulen, 1974; Lyons, 1973a, b). Planulae of *Anthopleura* possess 3 types of glandular cells in the ectoderm, and they exhibit the most complicated nervous system described to date for a planula larva (Chia and Koss, 1979). The nervous system consists of an apical organ, 1 type of endodermal receptor cell, 2 types of ectodermal receptor cells, inter-neurons, and a nerve plexus.

Werner (1973), in his analysis of the evolution of the cnidarian classes, proposed that the stem form of the recent cnidarians was a solitary, sessile, tetramerous polyp. He postulated that the Anthozoa were an early offspring from this common ancestor, and that the Scyphozoa, Hydrozoa, and Cubozoa arose from another evolutionary line. The acceptance of Werner's concept would result in the classification of the phylum Cnidaria into 2 subphyla: Anthozoa and Medusozoa. In the Anthozoa the polyp is the sexual adult and a medusa never develops, whereas, in the Medusozoa a medusa is the normal sexual adult and the polyp is regarded as a larval stage. The Medusozoa would consist of the extinct class Conulata and the recent classes Scyphozoa, Hydrozoa, and Cubozoa. Based on the comparative fine-structural morphology of the planulae examined to date, planulae of scyphozoans and hydrozoans appear to be more closely related to each other than are planulae of the scyphozoans and anthozoans or planulae of the hydrozoans and anthozoans (Koss, personal communication). The cells which comprise hydrozoan and scyphozoan planulae

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FIGURE 12. Transverse section of the supportive cells of the endoderm. Foot processes of these cells insert on the mesoglea. PAS-positive granules, vacuoles, glycogen particles, and electron-dense granules are found in the basal regions of the cells. The cells possess a single cilium surrounded by microvilli which projects into the gastrovascular cavity. The nucleus of the cell is centrally located. C = cilium; EC = ectoderm; GL = glycogen particles; MG = mesoglea; N = nucleus of supportive cell; Y = PAS-positive granules. Bar = 2 $\mu$m.

FIGURE 13. Transverse section of the gastrovascular cavity of a planula. Numerous cilia and microvilli from the endodermal supportive cells project into the lumen of the cavity. GV = gastrovascular cavity; MG = mesoglea. Bar = 5 $\mu$m.

are morphologically similar and are not as complex in their overall structural design as are the cells of anthozoan planulae. Some anthozoan planulae are provided with spirocysts which are absent in planulae of hydrozoans and scyphozoans. Furthermore, many anthozoan planulae possess an apical organ. Such a structure has not been reported in a hydrozoan or scyphozoan planula.

It is our judgment that in the future many more cnidarian biologists will turn their attention to the comparative cytology of planulae. It is expected that results from such investigations will contribute new ideas to both the developmental biology of the cnidarians and to the phylogenetic classification of the cnidarians.

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# REGIONAL DISTRIBUTION OF MUSCLE FIBER TYPES IN THE ASYMMETRIC CLAWS OF CALIFORNIAN SNAPPING SHRIMP

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## ABSTRACT

The properties of the opener and closer muscles in the asymmetric claws of *Alpheus californiensis* have been investigated using sarcomere length measurements and histochemical techniques. In the smaller pincer claw two types of muscle fibers are regionally distributed within the single closer muscle. A central band of fibers have short (2.5 μm) sarcomeres and high myofibrillar ATPase activity. Intermediate-type fibers have smaller diameters, sarcomeres 8.5 to 9 μm in length and low myofibrillar ATPase activity. The snapper closer muscle, by contrast, is composed of fibers with long (11–14 μm) sarcomeres and low myofibrillar ATPase activity. Opener muscle fibers in the pincer claw have shorter sarcomere lengths than their counterparts in the snapper claw.

## INTRODUCTION

In certain crustaceans, claw dimorphism is accompanied by an asymmetry of claw muscle properties. For example, in lobsters (*Homarus americanus*) the rapidly closing cutter claw has a large proportion of fast closer muscle fibers, while the slowly closing crusher claw is composed of a uniform population of slow muscle fibers (Govind and Lang, 1974; Lang *et al.*, 1977). In addition it has been shown recently that a similar asymmetry of fiber properties is present between the claw opener muscles (Govind *et al.*, 1981).

In the dimorphic claws of snapping shrimp (*Alpheus*) differences exist in claw closer muscle properties (Stephens and Mellon, 1979). In *A. heterochelis* and *A. armillatus* there are three muscles in each claw: a single opener, a minor closer, and a main closer muscle (Ritzmann, 1974). Analysis of sarcomere lengths, used as an indication of muscle fiber contraction properties (Atwood, 1973, 1976; Josephson, 1975), reveals that differences occur only in the main closer muscle. In the larger snapper claw the main closer muscle is composed of a uniform population of slow fibers with long (10–15 μm) sarcomeres. In the smaller pincer claw the main closer muscle has two populations of fiber types. Fast fibers in the central portion of the main closer muscle have relatively large diameters and short (2 and 3 μm) sarcomeres. Intermediate-type muscle fibers have sarcomeres that measure between 6 and 8 μm and are located on the medial and lateral margins of the muscle.

A fascinating feature of adult snapping shrimp is an ability to reverse claw configuration (Wilson, 1903; Przibram, 1931; Mellon and Stephens, 1978). Removal or denervation of the snapper claw causes the remaining pincer to become transformed into a new snapper claw, while a pincer regenerates at the site of the old snapper claw. Pincer–snapper transformation involves a change in the properties of the main closer muscle fibers from fast and intermediate in the pincer to slow in the snapper (Stephens and Mellon, 1979).

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Measurement of sarcomere length is one method used to examine the properties of single fibers in a particular muscle. A major disadvantage with this technique is the difficulty in constructing a complete picture of the properties of all of the fibers in a given muscle. A new technique, however, allows differentiation of fast and slow crustacean muscle fibers based on histochemistry (Ogonowski and Lang, 1979; Silverman and Charlton, 1980). In a previous paper we showed that in *A. californiensis* the sarcomere lengths of the single claw closer muscle are similar to those described for the main closer muscle of *A. heterochelis* (Stephens *et al.*, unpublished observations). In the present study we have used histochemistry and sarcomere length measurements to investigate the properties of the closer and opener muscles in dimorphic claws of *A. californiensis.*

## MATERIALS AND METHODS

Snapping shrimp (*Alpheus californiensis*) were obtained commercially from Venice, California, and were retained individually in constantly circulating, artificial sea water at 14°C. The animals were fed Tetramin twice weekly, and under these conditions lived for at least 3 months in the laboratory.

### *Sarcomere length measurements*

Sarcomere length measurements were made from opener or closer muscles in pairs of claws removed from adult shrimp. One of the claw muscles was carefully dissected away and the remaining muscle was fixed at resting length; the closer muscle was fixed with the dactyl in the open position, and the opener muscle with the dactyl in the closed position (Lang *et al.*, 1977; Stephens and Mellon, 1979). To prevent measurement errors due to muscle contraction (Meiss and Govind, 1979), the dissected claws were soaked for 60 minutes in a snapping shrimp saline in which calcium ions had been replaced with magnesium. The claws were then fixed for 2 days in alcoholic Bouin's solution. Individual muscle fibers were carefully teased apart in 80% ethanol on a microscope slide, and examined using a compound microscope fitted with Normarski optics. The length of five successive sarcomeres was measured using a calibrated filar ocular micrometer. At least three measurements were made for each muscle fiber and the average length of a single sarcomere was calculated.

### *Histochemistry*

Certain histochemical properties of the muscle fibers in the dimorphic claws were examined. Fully developed pincer and snapper claws were removed from adult shrimp, quickly frozen in liquid nitrogen, and sectioned on a cryostat microtome. Transverse sections (15 $\mu$m thick) from pairs of claws were mounted on glass cover slips and the myofibrillar adenosinetriphosphatase (ATPase) activity was determined using a technique used for lobsters (Ogonowski and Lang, 1979)—a modification of conventional methods for vertebrate tissue (Padykula and Hermann, 1955). A more recently published technique for determining myofibrillar ATPase activity (Silverman and Charlton, 1980) was employed with less success.

## RESULTS

### *Sarcomere length measurements*

Sarcomere length measurements were made from closer and opener muscle fibers in pairs of fully developed claws removed from the same animal. The closer

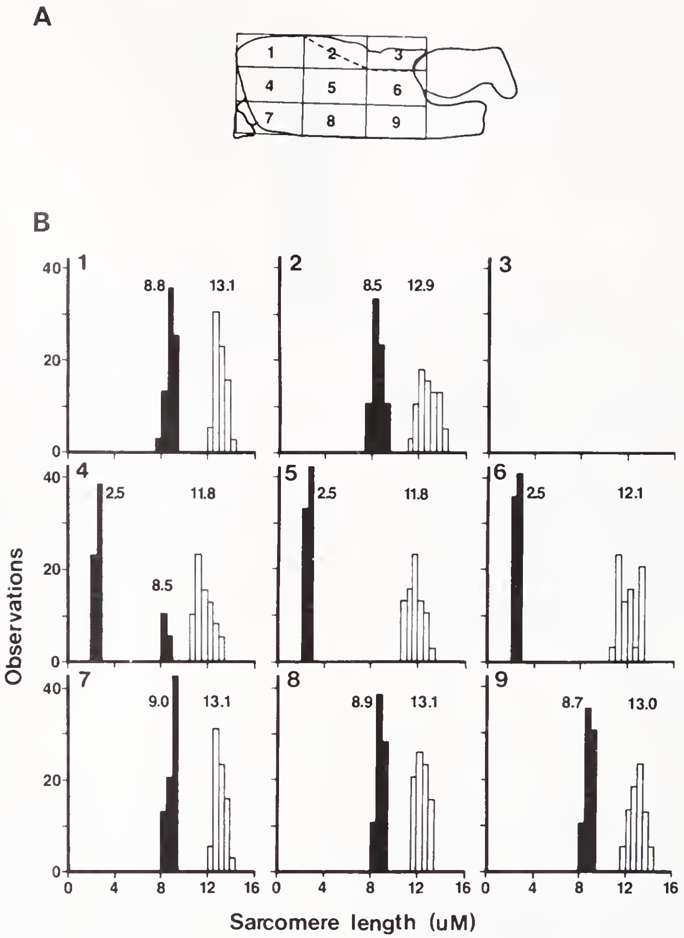

FIGURE 1. The regional distribution of sarcomere lengths in the claw closer muscle. (A) Diagram of a snapper claw with the propus divided into 9 regions. (B) Histograms of sarcomere length data for closer muscle fibers removed from each region (1–9) in a pincer (filled columns) and snapper (open columns) claw removed from the same animal. Inset numbers represent mean sarcomere length values.

muscle was divided by eye into 9 regions (Fig. 1A) and muscle fibers were carefully removed from the central portion of each region. It should be noted that observations were not made from fibers in region 3 since only opener muscle is present in that region. In the smaller pincer claw, closer muscle fibers located in the dorsal (1 and 2) and ventral (7 to 9) regions are made up of sarcomeres with mean lengths of 8.5 to 9.0 $\mu$m (Fig. 1B). The central regions (4 to 6), by contrast, contain muscle fibers with mostly short (2.5 $\mu$m) sarcomeres. A similar regional distribution of different fiber types has been reported for the pincer main closer muscle of *A. armillatus* (Stephens and Mellon, 1979).

In the larger snapper claw, the closer muscle fibers are composed of long sarcomeres (Fig. 1B). Fibers located in the central regions of the muscle have sarcomere lengths that are shorter than those in the dorsal and ventral regions, however these differences are not statistically different (Student's *t*-test).

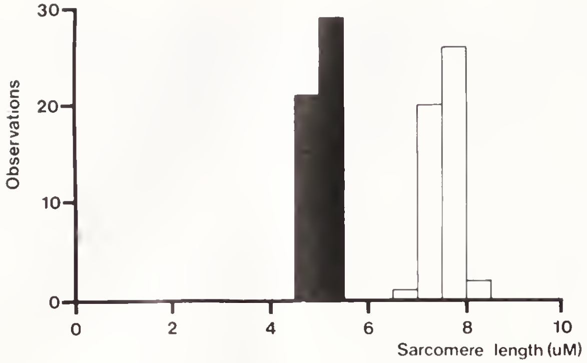

FIGURE 2. Histograms to show the sarcomere lengths of opener muscle fibers in a pair of pincer (filled columns) and snapper (open columns) claws from the same animal.

Opener muscle fibers from pincer and snapper claws are composed of sarcomeres of different lengths. In the example given in Figure 2, opener muscle sarcomeres have mean lengths of 7.5 $\mu$m and 5.0 $\mu$m, respectively, for the snapper and pincer. No regional differences in sarcomere length were observed in the opener muscle of either claw.

### *Histochemistry*

Figure 3 shows photomicrographs of frozen transverse sections taken from a pair of claws and stained for myofibrillar ATPase activity. Sections of snapper claws showed uniform light staining profiles for opener and closer muscle fibers (Fig. 3C). In sections of the pincer claw, however, a central band of closer muscle fibers was always darkly stained (Figs. 3D–F), indicating a higher ATPase activity in these fibers than in those located in the dorsal and ventral regions. Using this same technique on lobsters, Ogonowski and Lang (1979) showed that muscle fibers with high myofibrillar ATPase activity are rapidly contracting, fast muscle fibers. The location of the dark-staining closer muscle fibers in the pincer claw (Fig. 3) correlates well with the location of short sarcomere fibers (Fig. 1), indicating that there is a central band of fast fibers. In the pincer claw of *A. armillatus* the fast main closer muscle fibers have a larger diameter than the intermediate muscle fibers (Stephens and Mellon, 1979). In the present study myofibrillar ATPase activity was used to differentiate between fast and intermediate fibers in the pincer closer muscle. Figure 4 shows closer muscle fiber diameter data for the light- and dark-staining fibers in transverse sections of a pincer claw, and for closer muscle fibers in the contralateral snapper claw. Although there is no statistical difference between the diameters of the two types of pincer closer muscle fibers, it is apparent that the dark staining fibers in the central region of the claw have a slightly larger diameter than the light-staining fibers in the ventral and dorsal regions. Furthermore, the closer muscle fibers in the snapper are about twice the diameter of their counterparts in the pincer claw.

## DISCUSSION

In many crustacean neuromuscular preparations there is a correlation between the speed of muscle contraction, sarcomere length (Atwood, 1973, 1976; Govind

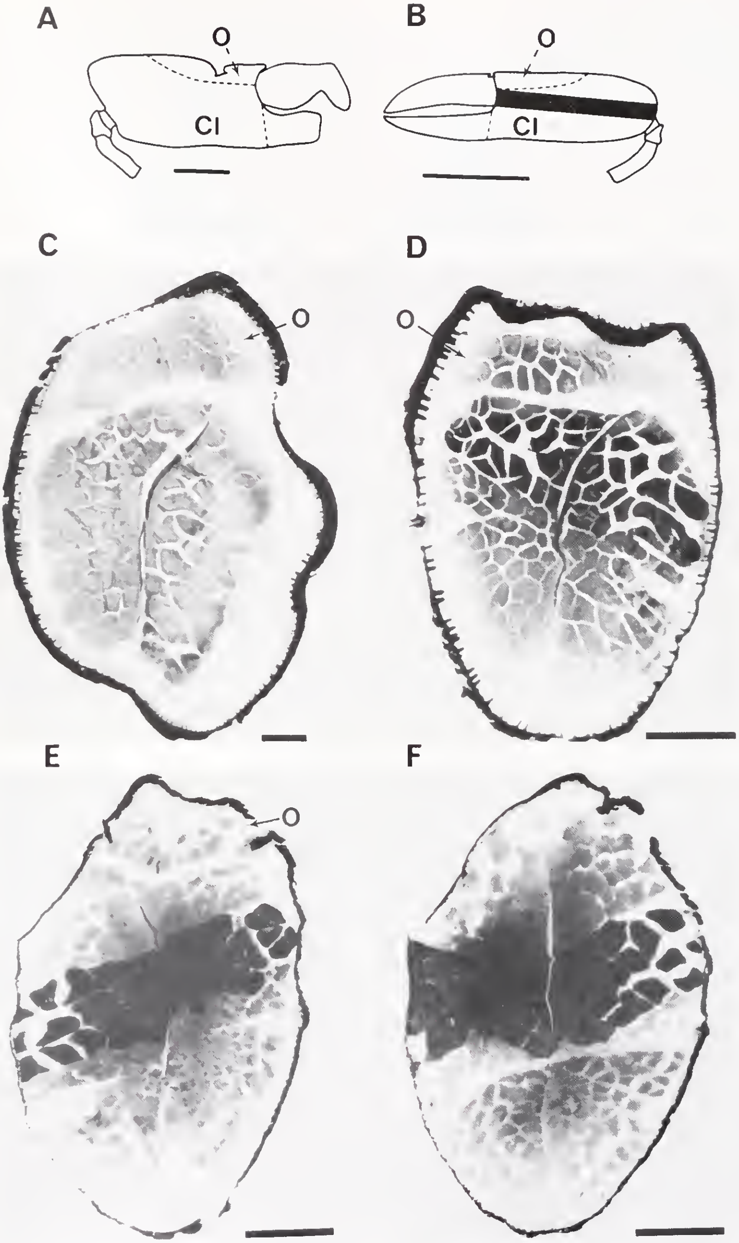

FIGURE 3. Myofibrillar ATPase activity of the claw muscles.

(A,B): Diagrams of a snapper (A) and pincer (B) claw showing the locations of the opener (O) and closer (Cl) muscles. The dark band in the pincer closer muscle represents the location of the fibers with high myofibrillar ATPase activity.

(C–F): Myofibrillar ATPase activity of the claw muscles in frozen transverse sections (15 $\mu$m thick) of a snapper (C) and a pincer (D–F) claw. A band of fibers with high myofibrillar ATPase activity is present in the pincer closer muscle. Sections D, E, F were taken distally, centrally, and proximally, respectively, through the propus of the pincer.

Calibration: 1mm (A,B) and 500 $\mu$m (C–F).

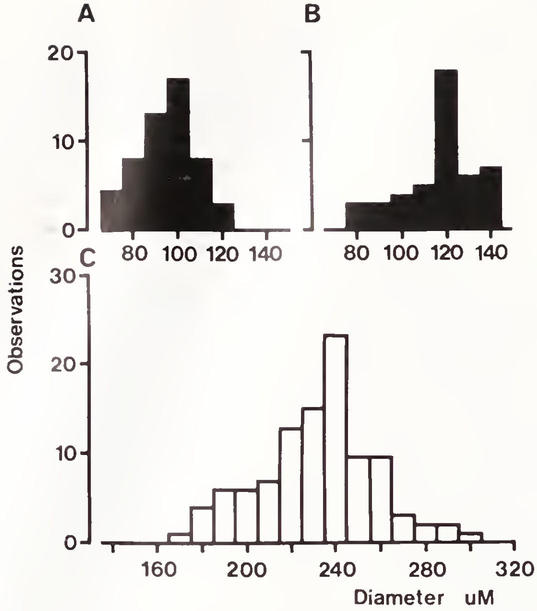

FIGURE 4. The diameter of closer muscle fibers in a pincer (A, B) and snapper (C) claw. Measurements were made from frozen transverse sections of a pair of claws. Data is given for pincer fibers with low ATPase activity (A), high ATPase activity (B), and for snapper closer muscle fibers.

and Lang, 1974; Josephson, 1975) and myofibrillar ATPase activity (Ogonowski and Lang, 1979; Ogonowski *et al.*, 1980; Silverman and Charlton, 1980). Rapidly contracting muscle fibers have short sarcomeres and high myofibrillar ATPase activity, while slow muscle fibers have long sarcomeres and low myofibrillar ATPase activity. Thus in the pincer claw of *A. californiensis*, the fibers in the central region of the closer muscle are presumably fast, while those located on the dorsal and ventral surfaces of the closer muscle are presumably intermediate speed fibers (Figs. 1 and 3). A histological examination of fixed claws from *A. armillatus* revealed similar results for the pincer main closer muscle (Stephens and Mellon, 1979). In addition, it was shown that the centrally located fast muscle fibers have a larger diameter than the intermediate fibers. The possibility that these centrally located muscle fibers contracted immediately prior to fixation, producing a decreased sarcomere length and an increased fiber diameter, could have produced erroneous results (C. Phillips, personal communication). However the present investigation, using frozen sections and also prolonged soaking in calcium-free saline prior to fixation to prevent muscle contraction, produced a similar regional distribution of closer muscle fiber types, without major differences in muscle fiber diameter. Furthermore, we have taken transverse sections of claws of *A. californiensis* following the procedure of Stephens and Mellon (1979) and have observed no clear regional differences in the diameter of pincer closer muscle fibers (unpublished observations). Moreover it is interesting that a distinct band of fast muscle fibers has been found in the central region of the closer muscles of both claws of larval homarid lobsters (Ogonowski *et al.*, 1980). During normal development the closer muscle of the larger crusher claw becomes uniformly slow, while the cutter claw closer muscle retains the dimorphism of fiber types in the adult (Lang *et al.*, 1977).

In the absence of direct measurements, histochemical and histological properties of muscle fibers can provide an indication of contraction speed. In many crustacean muscles, short sarcomere fibers with high myofibrillar ATPase activity are fast, while long sarcomere fibers with low ATPase activity are slow (Atwood, 1973, 1976; Josephson, 1975; Ogonowski and Lang, 1979; Silvermann and Charlton, 1980). From this evidence it appears that the pincer closer muscle is composed of fast and intermediate fibers, while the snapper closer muscle consists of fibers that contract slowly but produce large amounts of tension. This is consistent with behavioral observations made on snapping shrimp (Ritzmann, 1974; Schein, 1975). The pincer claw is used for manipulation of small objects while the snapper claw is used only during territorial encounters with conspecific shrimp. The dactyl initially moves to open the snapper claw and, in Californian snapping shrimp, a pair of discs on the propus and the dactyl become opposed (Ritzmann, 1973). The closer muscle then develops tension to overcome the adhesive force between the discs. The dactyl rapidly closes and causes a jet of water to be projected towards the intruder and also produces the characteristic snapping sound.

The sarcomere length values for the single snapper closer muscle of *A. californiensis* (Fig. 1B) are similar to those reported for the main closer muscle of *A. armillatus* (Stephens and Mellon, 1979). Furthermore, examination of *A. armillatus* with claws undergoing pincer–snapper transformation revealed that the fast and intermediate main closer muscle fibers in the pincer change to slow muscle fibers during this normal developmental process. If, in *A. californiensis*, the differences in the properties of the closer muscle fibers in pairs of claws represent the changes that take place as a pincer transforms into a new snapper, it is apparent that there are similar changes in the closer muscle fiber properties in the two species. However, we have shown recently that the differences in motor axon synaptic facilitation reported for *A. armillatus* (Stephens and Mellon, 1979) are not present in *A. californiensis* (Stephens *et al.*, unpublished observations). Examination of facilitation, using pairs of junctional and synaptic potentials evoked by stimulation of the excitatory axon, showed no facilitation in the snapper closer muscle. In fact, synaptic depression was recorded at short intervals (<100 ms). These data, together with the observation that the closer muscle fibers in either claw appear to be supplied by only one excitor axon, has raised the intriguing possibility that claw transformation may involve some reorganization of peripheral motor axon patterns, as seen in many vertebrate preparations (Rotshenker and McMahon, 1976; Brown and Ironton, 1977; Hubel *et al.*, 1977; Jackson and Diamond, 1979; Rotshenker and Reichert, 1980).

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# UREA PARTHENOGENETICALLY ACTIVATES THE CORTICAL REACTION AND ELONGATION OF MICROVILLI IN EGGS OF THE SEA URCHIN, *STRONGYLOCENTROTUS PURPURATUS*

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## ABSTRACT

Isotonic urea is believed to activate sea urchin eggs by triggering event(s) that normally follow cortical granule secretion at fertilization, particularly surface perturbations that result in elongation of microvilli (Mazia *et al.*, 1975). However, Moser (1940) reported that urea triggered the cortical reaction. Transmission electron microscopy showed that unfertilized *Strongylocentrotus purpuratus* eggs discharge their cortical granules in isotonic urea (containing 1.0 to 0.1 m*M* $CaCl_2$ or 25 m*M* EGTA) to form incipient fertilization envelopes and hyaline layers. These investments quickly disperse in urea. Elongation of microvilli follows cortical granule discharge. Urea-activated eggs can be fertilized after return to sea water and fail to elevate fertilization envelopes but do form hyaline layers. Hyalin must be secreted from a secondary reservoir in these eggs, since the cortical granule store is discharged during the prior urea activation. Cortical granule secretion and elongation of microvilli do not occur in urea plus 10 m*M* $CaCl_2$. These eggs form normal fertilization envelopes and hyaline layers when fertilized after return to sea water. Our results show that: (1) urea triggers an early event in sea urchin egg activation that stimulates cortical granule secretion; (2) cortical granule discharge precedes elongation of microvilli in urea-activated eggs as it does during normal fertilization; and (3) reduction or removal of external calcium is required for activation by urea.

## INTRODUCTION

Parthenogenetic agents, including isotonic non-electrolytes such as urea, have been used to study the sequence of events and causal relationships responsible for the activation of sea urchin eggs during fertilization (reviewed by: Loeb, 1913; Lillie, 1919; Allen, 1958; Epel, 1978; Schuel, 1978; Jaffe, 1980). On the basis of a scanning electron microscopic study performed on *Strongylocentrotus purpuratus* and *Lytechinus pictus*, it was suggested that urea bypassed the cortical reaction and activated the sea urchin egg by releasing a repressor component from its surface (plasma membrane and/or vitelline layer) and by inducing elongation of microvilli (Mazia *et al.*, 1975). Ammonia activation, which does not induce the cortical reaction (Loeb, 1913; Steinhardt and Mazia, 1973; Epel *et al.*, 1974), also was reported to promote elongation of microvilli (Mazia *et al.*, 1975). The concept advanced by Mazia's group appeared to fit with observations that: (1) elongation of microvilli normally occurs subsequent to secretion of the cortical granules during fertilization (Schroeder, 1979); (2) detachment of the vitelline layer from the egg's plasma membrane, a step

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in the assembly of the fertilization envelope (Schuel, 1978), is promoted by a protease secreted by the cortical granules (Longo and Schuel, 1973; Schuel *et al.*, 1973; Vacquier *et al.*, 1973); (3) urea removes the vitelline layer from unfertilized eggs (Moore, 1930); and (4) ammonia releases a surface glycoprotein that results in derepression of the egg's metabolism as normally takes place during fertilization (Johnson and Epel, 1975). The putative role of the release of repressor protein from the surface of ammonia-activated egg has been refuted (Carroll and Epel, 1981). Moreover, Mazia's group did not consider the possibility that urea can induce the cortical reaction, and that elongation of microvilli might be related to cortical granule exocytosis rather than to surface modifications. Urea had previously been shown to induce secretion of the cortical granules during parthenogenetic activation of *Arbacia* eggs (Moser, 1940).

The present study was undertaken to re-examine the effects of isotonic urea on the surface morphology of unfertilized *Strongylocentrotus purpuratus* eggs by means of transmission light and electron microscopy. A preliminary account has been presented previously (Schuel and Dandekar, 1981).

## MATERIALS AND METHODS

Specimens of the sea urchin *Strongylocentrotus purpuratus* were obtained from Pacific Bio-Marine Laboratories (Venice, CA) and maintained at 12–15°C in a marine aquarium (Aquarium Systems, Inc., Wickliffe, OH). Gametes were collected and stored as described previously (Schuel and Schuel, 1981). Only batches of eggs that yielded 95–100% fertilization in test insemination (0.1 ml eggs per 5 ml sea water plus 0.1 ml of 1% sperm) were used in this study. Experimental cultures were incubated at 15°C.

Artificial sea water was prepared from Instant Ocean salt mixture (Aquarium Systems, Inc.) and filtered through a $0.45\mu$ Millipore filter. Calcium-free sea water containing 25 m*M* EGTA (ethyleneglycol-bis($\beta$-amino ethyl ether)N,N'-tetra acetic acid) was prepared according to Detering *et al.* (1977). Isotonic urea (1.0 *M*) was prepared in deionized water, 10 m*M* $CaCl_2$, or 25 m*M* EGTA adjusted to pH 8.0 with NaOH. EGTA was obtained from Sigma Chemical Co., St. Louis, MO.

Unfertilized eggs were activated parthenogenetically by brief exposure to isotonic urea (Moser, 1940; Mazia *et al.*, 1975). Egg suspensions (1.0 ml) were added to 9.0 ml of urea and incubated for 60 sec. The eggs were then sedimented by gentle centrifugation (IEC Clinical Centrifuge) and the supernatant discarded. The eggs were resuspended in urea (to 10 ml), incubated for another 60 sec, sedimented again by centrifugation, and finally resuspended in sea water. This treatment took about 3 min. After exposure to the urea solutions, the eggs were inseminated and cultured in sea water. In some experiments eggs were observed for up to 5 minutes during a single continuous treatment with 9 parts isotonic urea plus 1 part sea water.

For morphological analysis, eggs were fixed with 3% glutaraldehyde in sea water. They were then processed for examination by transmission light and electron microscopy using previously described procedures (Longo and Anderson, 1972). Thin sections stained with uranyl acetate and lead citrate were examined with a JEOL-100B electron microscope. Thick sections stained with toluidine blue were examined by light microscopy.

## RESULTS

Live *Strongylocentrotus* eggs were observed by light microscopy during and following treatment with isotonic urea. Thin fertilization envelopes elevate from the surface of unfertilized eggs in the urea solution. Upon continued exposure to urea the fertilization envelopes recede toward the egg surface and become thinner, until in most cases no vestige of the fertilization envelope can be seen. These results are consistent with previous observations that urea parthenogenetically activates the cortical reaction in *Arbacia* eggs (Moser, 1940). When urea-activated eggs (two 60-sec washes) are returned to sea water they are indistinguishable from control eggs incubated in sea water. Urea-activated eggs can be fertilized. However, following insemination none of these eggs lift fertilization envelopes, but most form hyaline layers. Control eggs form normal fertilization envelopes and hyaline layers upon fertilization. Eggs fertilized following urea activation divide and develop at the same time as controls. About 10% of the urea-treated eggs fail to form hyaline layers after subsequent fertilization. These zygotes divide to form unorganized grape-like clusters of blastomeres during cleavage. These findings confirm previous observations by Moore (1930).

The effects of urea treatment on the surface morphology of *Strongylocentrotus* eggs was determined by light (data not shown) and electron microscopic (Figs. 1 and 2) analysis of fixed and sectioned specimens. Cortical granules are located subjacent to the plasma membrane in unfertilized (control) eggs (Fig. 1A). The vitelline layer is attached to the outer surface of the egg's plasma membrane, and short microvilli are present at the egg surface. Upon exposure to isotonic urea (9 parts plus 1 part sea water) the cortical reaction is triggered and results in lifting of the fertilization envelope (Fig. 1B). Patches of "hyalin-like" material are seen in the perivitelline space, but an organized hyaline layer does not form. The fertilization envelopes begin to fragment and disperse upon continued exposure to urea (data not shown). Examination of eggs returned to sea water after two 60-sec washes in isotonic urea reveals that the treatment completely removes the fertilization envelopes (Fig. 2A). Elongate microvilli are prominent features at the surface of these eggs. External investments (vitelline layer/fertilization envelope and hyaline layer) can not be detected outside of the eggs. After these eggs are fertilized, they form normal hyaline layers but do not form fertilization envelopes (Fig. 2B).

Several other aspects of the responses of urea-activated eggs were observed. When eggs are suspended in urea the fertilization envelope appears to lift simultaneously from the entire circumference of the activated eggs. Examination of fixed and sectioned specimens indicates that in each individual urea-activated egg the cortical reaction is at the same stage around the entire circumference (data not shown). By contrast, during normal fertilization the cortical reaction and the elevation of the fertilization envelope start at the site of attachment of the fertilizing sperm and spread around the surface of the egg (Moser, 1939a; Anderson, 1968). The incidence of eggs in the population that show a cortical reaction increases with exposure time to urea (Fig. 3). The data are presented in the form of a first order decay plot of unreacted eggs *vs* exposure time, from which the half time for urea activation can be estimated to be about 90 sec.

The release of calcium from internal stores is believed to play a critical role in the initiation of cortical granule exocytosis in sea urchin eggs during fertilization and upon parthenogenetic activation (reviewed by: Epel, 1978; Schuel, 1978; Jaffe, 1980). Accordingly we studied the effects of calcium on the parthenogenetic induction of the cortical reaction by urea. The normal calcium concentration of sea water is 10 m*M* (Cavanaugh, 1964). In the urea-activation experiments described above (Fig. 2), calcium is reduced to 1.0 m*M* in the first wash and 0.1 m*M* in the second wash. The urea solutions used by Mazia's group (1975) to induce elongation of microvilli contained 0.1 m*M* calcium. We found that when 25 m*M* EGTA is added

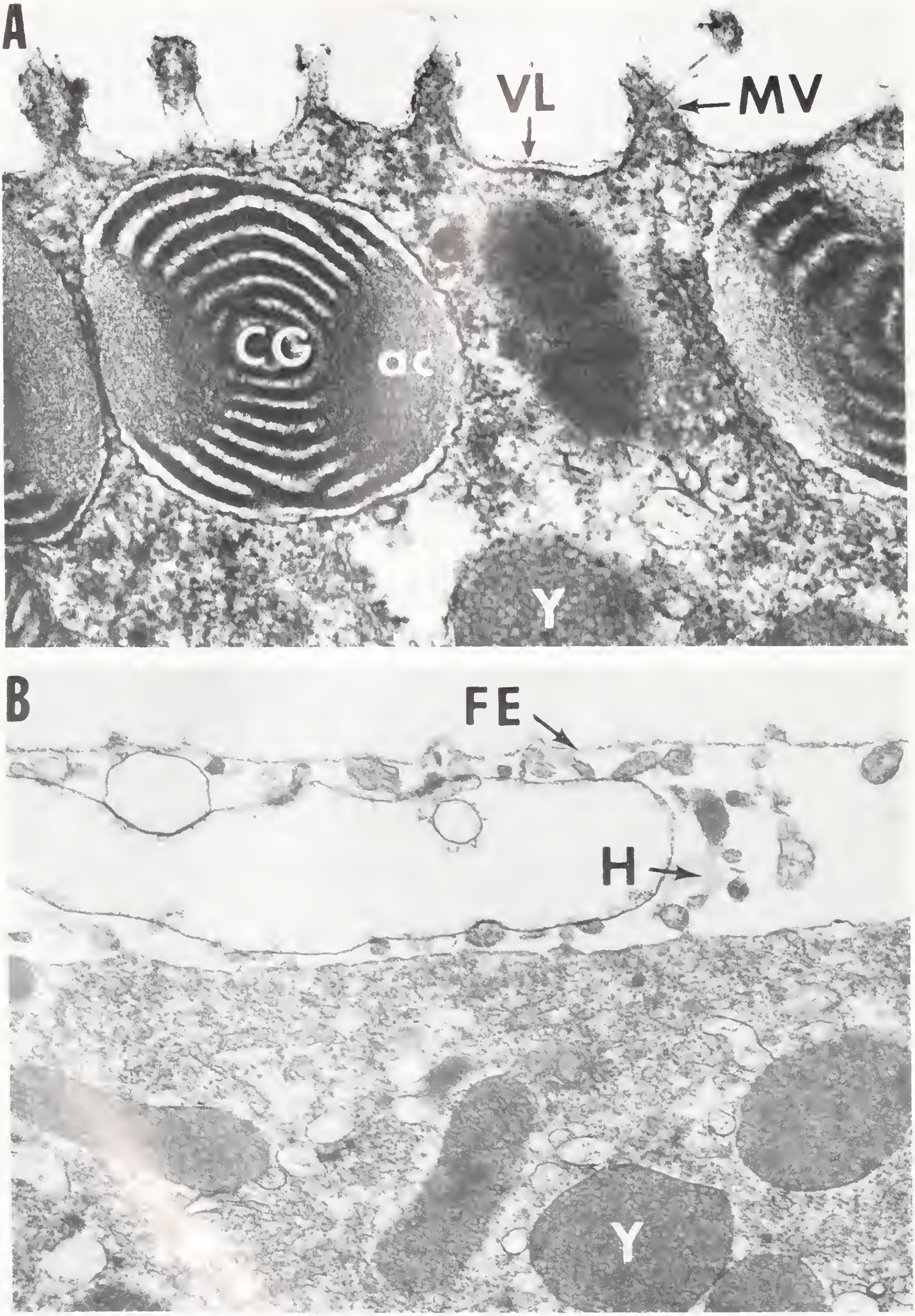

FIGURE 1. Electron micrographs showing parthenogenetic induction of the cortical reaction in unfertilized *Strongylocentrotus* eggs by isotonic urea.

*A*: Control egg in sea water. Cortical granules (CG) located just below the egg's plasma membrane show the amorphous (ac) and electron-dense spiral lamellae components characteristic of this species.

to the isotonic urea, cortical granule discharge occurs followed by elongation of microvilli as described above. Control eggs incubated under similar conditions in calcium-free sea water containing 25 m*M* EGTA do not show a cortical reaction (data not shown). Under these conditions EGTA reduces the free calcium in the culture solutions to below $10^{-7}$ *M* (Portzehl *et al.*, 1964). Conversely, cortical granule secretion and elongation of microvilli do *not* take place when unfertilized eggs are exposed to isotonic urea containing 10 m*M* calcium (Fig. 4A). When these eggs are fertilized after return to sea water, they undergo a normal cortical reaction to produce fertilization envelopes and hyaline layers (Fig. 4B). The thickened tri-laminar fertilization envelope shows sharp "tent-like" projections indicative of structuralization by secreted cortical granule contents in *Strongylocentrotus* (Veron *et al.*, 1977; Schuel *et al.*, 1982). These observations confirm previous findings by Moore (1930) that inclusion of calcium in the urea solutions protects the egg's capacity to form a fertilization envelope upon insemination after return to sea water.

## Discussion

The results of the present study show that, contrary to previous suggestions (Mazia *et al.*, 1975), urea does not mimic the presumed effects of ammonia in activating sea urchin eggs by triggering events that normally occur subsequent to the cortical reaction. Instead urea triggers discharge of the cortical granules. Furthermore, exocytosis of the cortical granules precedes elongation of microvilli in urea-activated eggs just as it does during normal fertilization. These findings confirm and extend earlier observations by Moser (1940). Although we did not examine their effects, Moser also noted that other non-electrolytes (glycerol, thiourea, and sucrose) elicited the same kind of visible cortical response as urea.

Elongation of microvilli during fertilization or upon parthenogenetic activation is a complex process that depends in part upon the insertion of the limiting membrane of the discharged cortical granules into the egg's original plasma membrane as a result of exocytosis (Schroeder, 1979) as well as the polymerization of actin in the cortex to form bundles of microfilaments (Burgess and Schroeder, 1977; Carron and Longo, 1982). Urea appears to mimic other parthenogenetic treatments such as hypertonic sea water (Sachs and Anderson, 1970) and calcium ionophore A23187 (Chambers and Hinkley, 1979; Carron and Longo, 1982) which induce elongation of microvilli as sequalae to cortical granule exocytosis. Elongation of microvilli in the absence of cortical granule exocytosis can be induced by application of hydrostatic pressure immediately after insemination (Chase, 1967; Hylander and Summers, 1982) and by treating unfertilized eggs with papain (Spiegel and Spiegel, 1977). The belief that ammonia and urea induce microvillar elongation in the absence of prior cortical granule exocytosis (Mazia *et al.*, 1975) appears to be erroneous. Other workers who examined ammonia-activated eggs by transmission electron microscopy found that the microvilli do not elongate and the cortical granules do not secrete (Nicotra and Arizzi, 1979; Hylander and Summers, 1981; Carron and Longo, 1982; Schuel and Dandekar, unpublished data). Cortical granule exocytosis is sometimes seen during ammonia activation (Carroll and Epel, 1981). However, Mazia's group (1975) did not determine whether cortical granule exocytosis had occurred in urea or ammonia activated eggs.

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The vitelline layer (VL) is closely applied to the outer surface of the plasma membrane. Note the short microvilli (MV). Yolk platelet (Y). 50,000×.

*B*: Activated egg fixed during exposure to 9 parts isotonic urea and 1 part sea water. The cortical granules have discharged and a thin fertilization envelope (FE) has elevated over the egg surface. A patch of "hyalin-like" material (H) is present in the perivitelline space. Yolk platelet (Y). 33,300×.

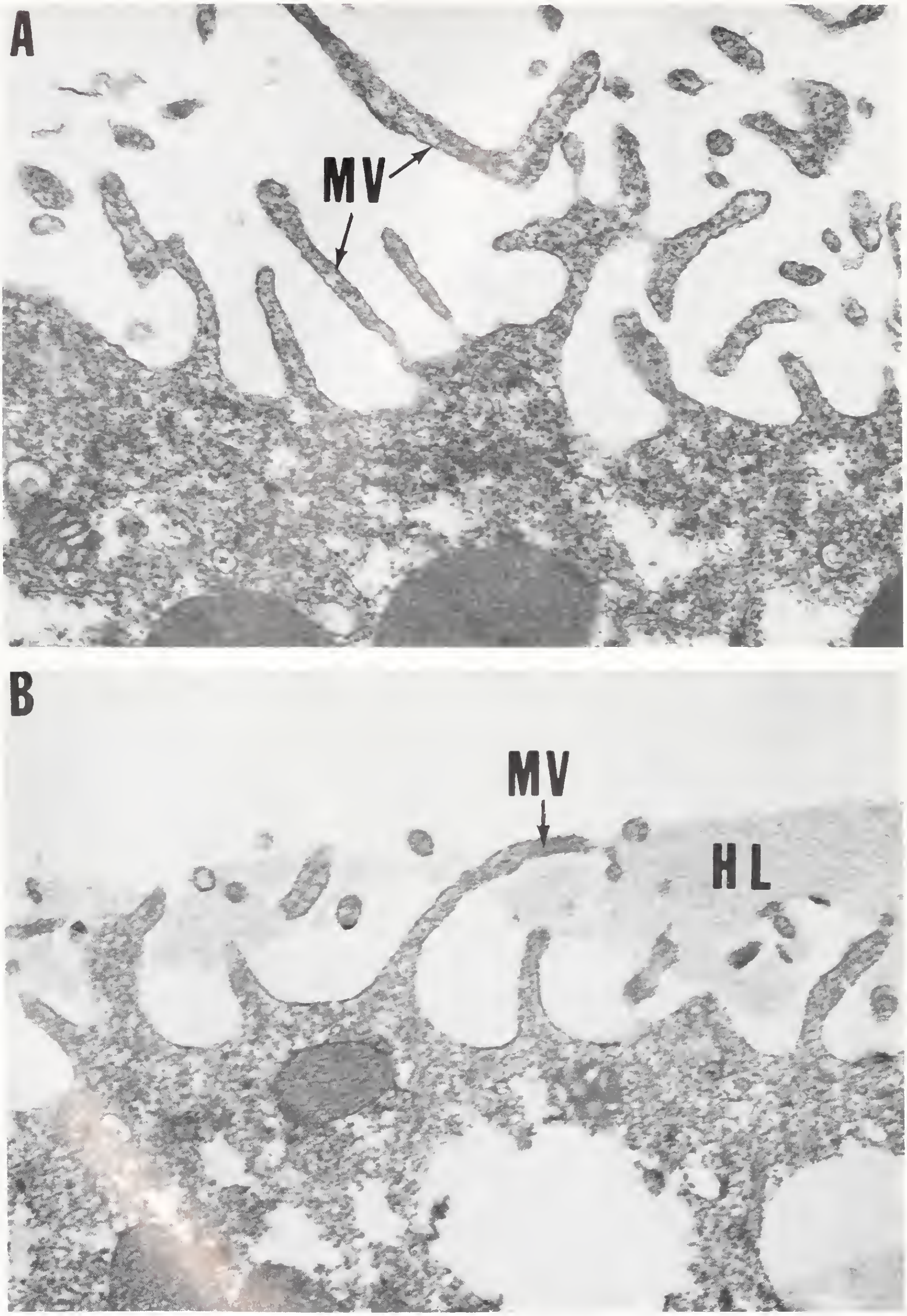

FIGURE 2. Electron micrographs showing the formation of the hyaline layer in urea-activated eggs that are fertilized after return to sea water. 33,300×.

*A*: Egg washed twice with isotonic urea and fixed immediately after return to sea water. Note the numerous elongate microvilli (MV) and the absence of cortical granules.

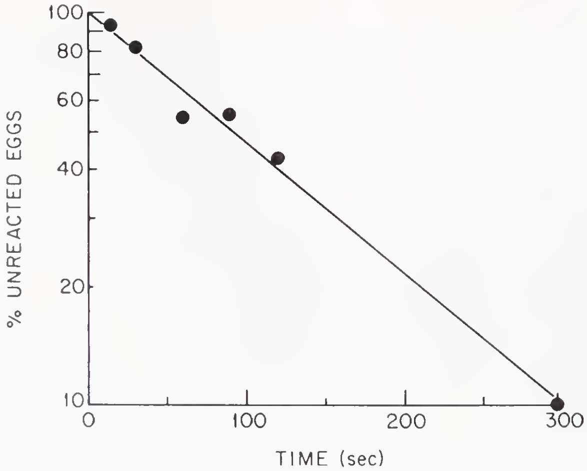

FIGURE 3. Effect of exposure time on incidence of cortical reaction in eggs activated by urea. The eggs (1.0 ml) were exposed to 9.0 ml of isotonic urea and fixed at indicated times. The incidence of reacted and unreacted eggs was scored from thick sections observed by light microscopy.

Morphological (Endo, 1961; Anderson, 1968) and biochemical (Kane, 1970) observations suggest that hyalin, the major structural protein of the hyaline layer (Stephens and Kane, 1970; Citkowitz, 1971), is secreted by the cortical granules during fertilization (reviewed by Schuel, 1978). In addition a secondary cytoplasmic reservoir that normally is slowly released during embryogenesis also is present in unfertilized eggs (Kane, 1973). These concepts have become controversial because McBlaine and Carroll (1980) claimed to show that hyalin is a cryptic protein on the surface of unfertilized eggs. The issue has been resolved by recent immunocytochemical studies using monospecific antibodies against pure hyalin (Hylander, 1981; Hylander and Summers, 1982; McClay and Fink, 1982). They found that hyalin is not detectable on the surface of eggs prior to secretion of the cortical granules, and is sequestered within cortical granules of unfertilized eggs. At the ultrastructural level hyalin is localized to the amorphous component of *Strongylocentrotus* cortical granules (Hylander, 1981; Hylander and Summers, 1982). The secondary hyalin reservoir is stored in small cytoplasmic vesicles (Hylander, 1981; Hylander and Summers, 1982). In the present study the hyaline layer formed by eggs that are fertilized subsequent to urea activation must have been secreted by the secondary reservoir, since the cortical granule store was discharged and dispersed while the eggs were being pretreated with urea. Hence this treatment could be used to collect hyalin from its two cytoplasmic reservoirs for further study.

Isotonic urea has been used to remove the vitelline layer from unfertilized sea urchin eggs (Moore, 1930) and the soft (non-cross-linked) fertilization envelope from fertilized eggs prior to the completion of hardening (Schuel *et al.*, 1982). When urea is applied to unfertilized eggs, it induces both the cortical reaction as well as the dispersal of the elevated fertilization envelope, and does not simply remove the vitelline layer as previously believed (Mazia *et al.*, 1975). Although the urea-activated egg remains receptive to sperm, its plasma membrane has been altered by cortical

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*B*: Urea-activated egg that was fertilized immediately after return to sea water. Fixed 10 min after insemination. Note the elongate microvilli (MV) embedded in the hyaline layer (HL) that invests the egg surface and the absence of the fertilization envelope.

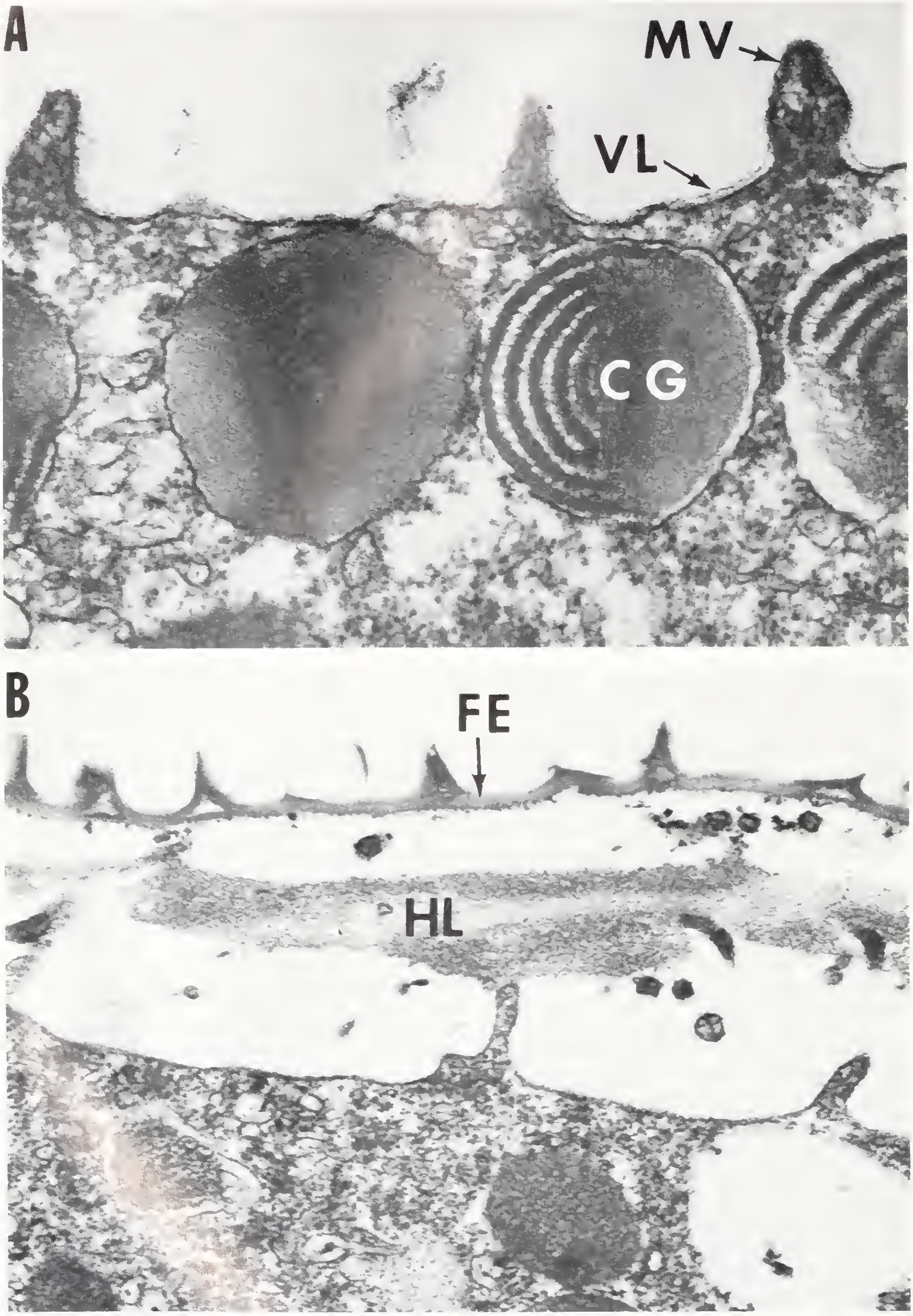

FIGURE 4. Absence of cortical reaction in eggs treated with isotonic urea containing 10 m$M$ $CaCl_2$. *A*: Egg washed twice (60 sec each) with isotonic urea containing 10 m$M$ $CaCl_2$, and fixed immediately after return to sea water. The cortex of this egg is identical to that of control eggs kept in sea water

granule exocytosis and elongation of microvilli to resemble that of a naked fertilized egg.

The effects of calcium on initiation of cortical granule secretion by urea are paradoxical. Certain other chemical and physical treatments that parthenogenetically trigger the cortical reaction in sea urchins require external calcium (Moser, 1939b). Also, the release of calcium from an internal store is thought to be part of the trigger mechanism for cortical granule exocytosis at fertilization or parthenogenetic activation (Steinhardt *et al.*, 1977; Zucker *et al.*, 1978). Calcium is stored at several sites (vitelline layer, plasma membrane, limiting membranes of cortical granules and other cytoplasmic organelles) in unfertilized eggs (Cardasis *et al.*, 1978), although the identity of the store that is released at fertilization is unknown. Urea appears to trigger the release of calcium from the same store that normally is released at fertilization (Zucker *et al.*, 1978). Yet the results of the present study show that urea elicits cortical granule secretion only when the external calcium is reduced. Taken together these findings possibly suggest that the removal of calcium from binding sites at the egg surface, perhaps the vitelline layer or plasma membrane, may be a prerequisite for the release of an internal store to trigger exocytosis. This feature of the response of sea urchin eggs to urea activation may provide a unique opportunity to study the initial actions of calcium in stimulus-secretion coupling and activation of development. Alternatively it is possible that the calcium level in normal sea water renders the unfertilized egg impermeable to urea and thereby inhibits parthenogenetic activation by the non-electrolyte. Additional work is required to answer these questions.

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(compare with Fig. 1A, above). Note the intact cortical granules (CG), vitelline layer (VL) applied to the egg's plasma membrane and the short microvilli (MV). 50,000×.

*B*: Isotonic urea-10 m*M* $CaCl_2$ treated egg that was fertilized immediately after return to sea water. Fixed 10 min after insemination. Normal fertilization envelope (FE) and hyaline layer (HL) have been formed. 33,300×.

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# EFFECT OF TEMPERATURE AND SALINITY ON LARVAL DEVELOPMENT OF SIBLING SPECIES OF *ECHINASTER* (ECHINODERMATA: ASTEROIDEA) AND THEIR HYBRIDS

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## ABSTRACT

Adult *Echinaster* Type 1 and Type 2 were collected along the west coast of Florida (25°C, 32‰S) and induced to spawn in the laboratory. Two-day old larvae of Type 1, Type 2, and their hybrids were subjected to temperature (T) and salinity (S) combinations (20, 25, 30°C; 25, 32, 39‰S). Response surface isopleths indicate that Type 1 and Type 2 larvae exhibit different developmental and growth rates in response to T/S combinations. Salinity was a dominant factor affecting development and growth. High and low salinities inhibited spine development. Developmental rates were directly related to temperature. Type 2 larvae exhibited a greater tolerance to temperature changes than did Type 1. Hybrids showed intermediate development and growth responses at the apparent optimal conditions and exhibited maternal characteristics.

## INTRODUCTION

Sibling species (Campbell and Turner, 1979) of the asteroid *Echinaster* occur in the vicinity of Tampa Bay, Florida. One is blue-orange in color and the other orange-brown. Downey (1973) characterized both as morphs of *E. modestus* based on spination in dried specimens. However, Campbell and Turner (1979) noted morphological differences between the two and concluded that they were sibling species. Other characteristics of these species are reviewed by Scheibling and Lawrence (1982).

The species have been classified by Atwood (1973) according to egg type: Type 1 with buoyant eggs and larvae which are planktonic for approximately two days, Type 2 with demersal eggs and completely benthic larvae. Type 1 adults generally spawn in the spring 2–4 weeks after Type 2 (Scheibling and Lawrence, 1982). Although these *Echinaster* species are found in different habitats, they may potentially interbreed.

Phenotypic variation among individuals may reflect basic genetic differences or environmentally induced modifications and phenotypic plasticity (Marcus, 1980). Genetic differences between the two species of *Echinaster* may be reflected in their physiology. The genetic and environmental factors that control the growth and development of the species may be distinguished by rearing individuals under controlled laboratory conditions.

Hybridization of these species of *Echinaster* is possible by cross-fertilization in the laboratory (Scheibling, 1982), although there is no evidence that hybridization of the two morphs occurs in nature. Echinoderm hybridization between both genera and species has been documented in the laboratory (Tennent, 1910; Harvey, 1956; Hägstrom and Lönning, 1961; Hinegardner, 1967, 1975; Horstadius, 1973; Lucas

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and Jones, 1976; Strathmann, 1981) and in the field (Verrill, 1909; Hägstrom and Lönning, 1961; Strathmann, 1981). Genetic differences may be reflected by differential responses of the species and their hybrids to different temperatures and salinities. The purpose of this study was to examine the effects of temperature and salinity on the development and growth of sibling species of *Echinaster* and their hybrids.

## MATERIALS AND METHODS

Individuals of *Echinaster* were collected in May and June, 1981 from the coastal waters of the eastern Gulf of Mexico: Type 1 from the Skyway Bridge at the mouth of Tampa Bay, Type 2 from the intercoastal waterway at Anna Maria Island. Individuals were maintained in the laboratory for 24 hours in aerated sea water at field temperature and salinity of 25°C and 32‰ S. Spawning was induced with 0.001 *M* 1-methyladenine in sea water either *in vivo* via intracoelomic injection, or *in vitro* using excised ovaries and testis. Eggs and sperm were pooled separately from 3–6 individuals. Eggs of each Type were both fertilized and cross-fertilized to produce 4 groups of zygotes: *Echinaster* Type 1 wild (Type 1♀ × ♂), *E.* Type 1 maternal hybrid (Type 1♀ × Type 2♂), *E.* Type 2 wild (Type 2♀ × ♂), and *E.* Type 2 maternal hybrids (Type 2♀ × Type 1♂). Eggs of each morph were fertilized less than two weeks prior to spawning in nature. The lecithotrophic embryos were maintained at field temperatures and salinities during early cleavage and gastrulation. After two days they developed into motile, modified brachiolarian larvae and were placed in experimental temperatures and salinities.

Larvae were subjected to temperature and salinity combinations in a 3 × 3 factorial design (20, 25, 30°C; 25, 32, 39‰ S). Ranges of temperatures and salinities experienced by *Echinaster* in the field are 10–33°C and 25–35‰ S. Preliminary experiments indicated that larval densities of 5–25 individuals per bowl had no effect on development and growth. Duplicate sets of 20 larvae were placed in 6 cm diameter glass bowls containing approximately 25 ml of filtered (0.45 $\mu$m Millipore filter) sea water at each temperature and salinity combination. Only one set of Type 2 hybrid larvae was used. Treatment salinities were obtained by the addition of distilled water or sea-salt to sea water. Temperature and salinity were controlled in constant environment chambers within 0.5°C and 1‰ S of selected values. Larvae were reared in the dark and water was changed daily. Treatment bowls were placed on rotators to insure adequate mixing.

The developmental stage of all larvae was monitored daily. Data represent pooled observations. The following stages were recorded: brachiolarian larvae, first appearance of the first and second pairs of tube feet, first appearance of the third pair of tube feet, first appearance of the fourth pair of tube feet, and the appearance of the mouth. All stages have been described and illustrated (Kempf, 1966; Atwood, 1973). In Figures 1 and 2 data represent the time of development reached by 75% of all individuals in a T/S combination. Radii of larval discs were measured at several stages. Growth was found to be linear by regression analysis. Average growth was measured by $\Delta$ radius/$\Delta$ time (days) × 100 (n = 20).

Response surface techniques (see Alderdice, 1972) were employed for statistical analysis of the effects of temperature and salinity combinations on larval development and growth. Isopleths were determined by a general linear model contained in the Statistical Analysis System (SAS). Approximate $r^2$ values between 0.81 and 0.98 indicate the data fit this model. Isopleths were extrapolated by the computer over the ranges of temperatures and salinities tested.

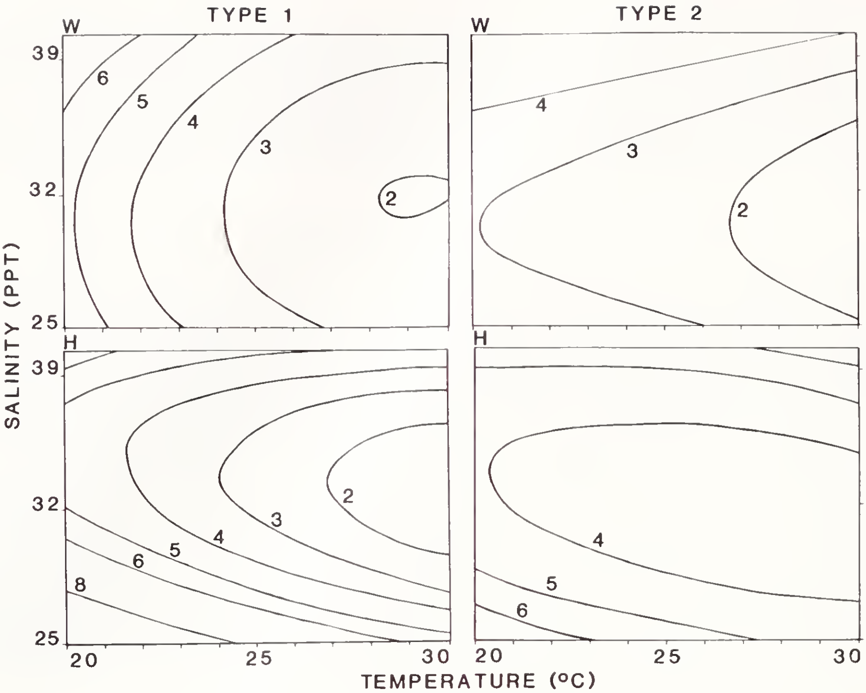

FIGURE 1. Response surface estimates of time (days) until the appearance of the first and second pairs of tube feet. Left column represents *Echinaster* Type 1 wild (W) and hybrid (H) individuals. Right column represents Type 2 wild and hybrid individuals.

## RESULTS

Response surface models of temperature and salinity combinations on the time (days) until the appearance of the first and second pairs of larval tube feet for *Echinaster* Type 1, Type 2, and their hybrids are shown in Figure 1. The appearance of the first and second pairs of tube feet represent an early stage of development of the larvae. Type 2 wild developed faster than Type 1 wild. The apparent optimal conditions, *i.e.*, those resulting in the fastest rates of development, are not different at this stage. Small changes in salinity appear to have a pronounced effect on development, indicated by narrow isopleths. Hybrids show intermediate developmental rates at the apparent optimal conditions with the maternal influence being strongest. Hybrids developed more slowly than the maternal parent at the extreme temperature and salinity combinations.

Response surface models of temperature and salinity combinations on time (days) until the appearance of the mouth are shown in Figure 2. This represents a late stage in the development of the juvenile just prior to feeding. The mouth of *Echinaster* Type 1 wild generally appears earlier than Type 2 wild. Distinct differences were seen in the apparent optimal conditions. Hybrids generally exhibit a dominant maternal influence. At this stage Type 1 hybrids exhibited faster rates of development than the maternal parent, and Type 2 hybrid rates were slower than the maternal parent.

Response surface models of temperature and salinity combinations on growth rates are shown in Figure 3. Growth rates were faster in Type 2 individuals and

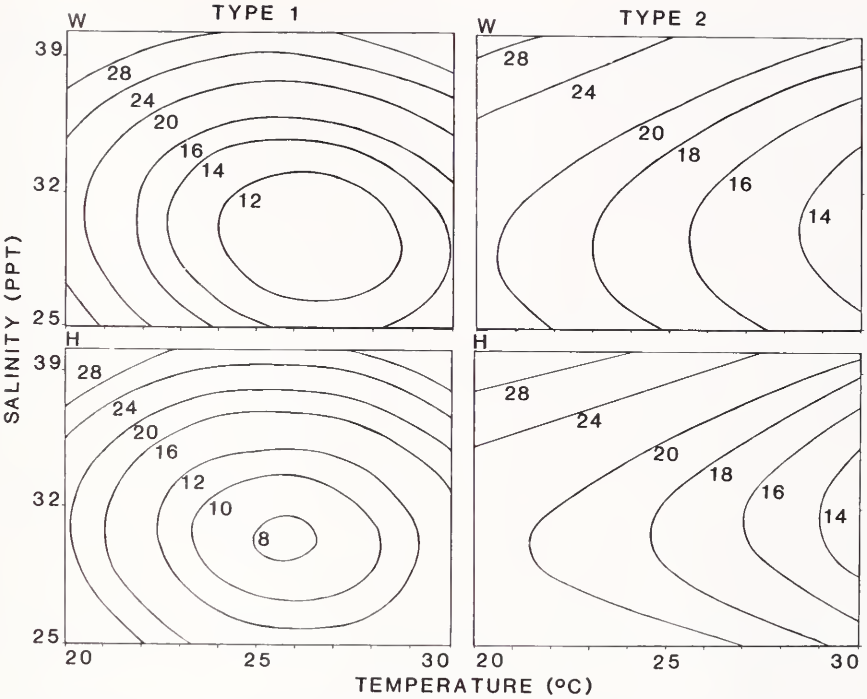

FIGURE 2. Response surface estimates of time (days) until the appearance of the mouth. Left column represents *Echinaster* Type 1 wild (W) and hybrid (H) individuals. Right column represents Type 2 wild and hybrid individuals.

correspond with faster development (Figures 1 and 2). Apparent optimal conditions differed between Type 1 and Type 2 wild. Salinity changes had a pronounced effect on growth. Isopleths along the temperature axis indicated the eurythermality of both types, with Type 2 being more eurythermal than Type 1. Maternal hybrids exhibited intermediate growth rates at the optimal temperature and salinity combinations.

Initial exposure of the larvae to T/S combinations induced negligible mortality in all combinations (<1%). Greatest mortality (20%) occurred after larvae developed the mouth, and was presumed to result from lack of food. There was no higher mortality among hybrids.

Abnormal spine development was observed in several larvae exposed to high and low salinities. In these organisms there was little or no development of mouth-frame armature spines or of the terminal spines of the rays, as observed by light microscopy. Several larvae exposed to high salinity exhibited abnormal ray number development.

## DISCUSSION

Temperature and salinity are of primary importance in determining larval development and survival in marine habitats. The importance of the combined effects of temperature and salinity has been emphasized by Kinne (1970). These factors are particularly important to larvae that may encounter a variable environment, as the range of environmental factors tolerated by larvae and juveniles is usually narrower than that tolerated by the adult (Vernberg and Vernberg, 1975).

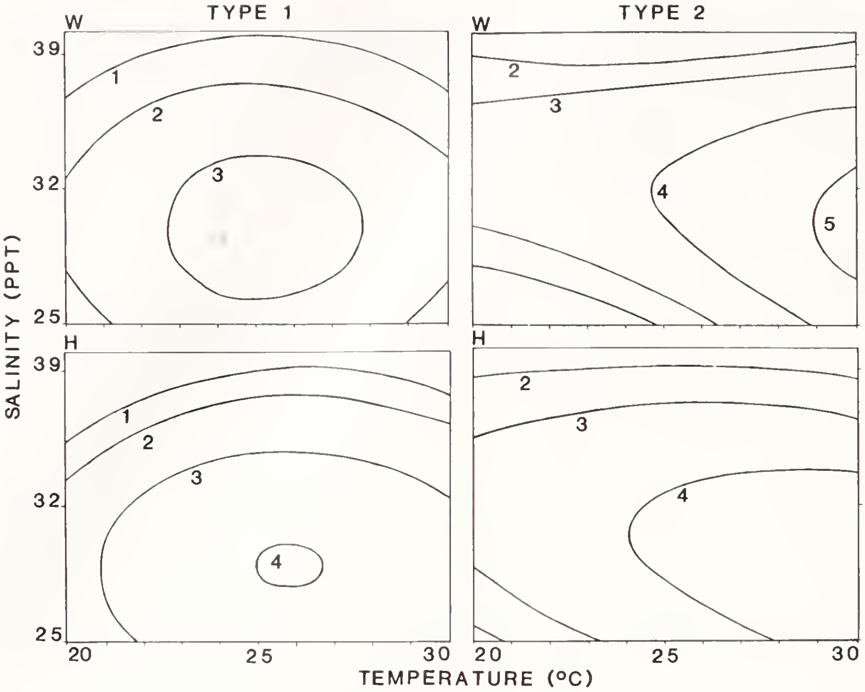

FIGURE 3. Response surface estimates of growth rates. Large values indicate faster rates of growth. Left column represents *Echinaster* Type 1 wild (W) and hybrid (H) individuals. Right column represents Type 2 wild and hybrid individuals.

Within the thermal tolerance limits of the species, warmer temperatures normally accelerate the growth processes. Development rates of larval *Echinaster* Types 1 and 2 were directly related to temperature. This was expected, as metabolic rate increases with temperature in larvae of marine invertebrates in general (Kinne, 1970).

Salinity greatly influenced development in *Echinaster* Types 1 and 2. Optimal development and growth of both Types 1 and 2 occurred at 28–32‰ S. In both types poor spine development occurred in many individuals exposed to low and high salinities. In many individuals there was little or no development of the mouth-frame armature spines or of the terminal spines of the rays. This indicates that salinity may affect the deposition of carbonate material during spine formation. Metabolism associated with carbonate deposition may be sensitive to changing salinities and not due to the concentrations of $Ca^{++}$ and $Mg^{++}$ in the water, as poor spine formation occurred at both low and high salinities. In addition, several larvae exposed to high salinities exhibited abnormal ray number development, *i.e.*, 3- or 4-rayed instead of the usual 5 (Watts *et al.*, 1983).

The first and second tube feet pairs developed earlier in Type 2 wild individuals than Type 1 individuals. This may be adaptive to Type 2 larvae which generally inhabit shallow-water seagrass beds subjected to wave action. Apparent optimal temperatures and salinities between Type 1 and 2 do not differ at this early stage of development. However, low temperature has a more pronounced effect on Type 1 than Type 2 as indicated by slower developmental rates in Type 1 larvae.

The mouth generally developed earlier in Type 1 wild individuals than in Type 2. The mouth in Type 1 appears after the third pair of tube feet while the mouth in Type 2 does not appear until after the fourth pair of tube feet. The differences in developmental rates are similar to those found by Atwood (1973) and Kempf (1966). Distinct differences were seen in the apparent optimal temperatures and salinities at this stage. At high temperatures, Type 2 individuals developed faster than did Type 1, and these differences are also shown in faster growth rates of Type 2 larvae. However, once the mouth is present and the juveniles begin to feed, Type 1 grows more rapidly than Type 2 (Scheibling, 1982).

Type 2 appears to be more eurythermal than Type 1 and this may be reflective of its shallow water existence. Type 1 generally inhabits deeper water than Type 2 (Atwood, 1973). The greater tolerance of Type 2 for a wider range of temperatures in shallow water may be related to past selective influences by the thermal environment on previous generations. Larval development and growth rates may also be influenced by environmental conditions experienced by the adults, particularly during gonadal development (Davies, 1958). Thermal tolerance of aquatic poikilotherms is in part dependent on their thermal history (Kinne, 1970).

Hybrids of *Echinaster* Type 1 and Type 2 generally exhibited intermediate developmental and growth rates at the apparent optimal temperatures and salinities. Type 1 hybrids at time of the appearance of the mouth exhibited faster developmental and growth rates than the maternal parent, while Type 2 hybrids exhibited slower developmental and growth rates than their maternal parent. This indicates that developmental and growth rates are directly related, but separate phenomena.

These factors suggest that the differences in temperature and salinity responses between the two morphs are genetically controlled, and are not environmentally influenced. The maternal influence is apparently stronger in the hybrids than the paternal influence. Andronikov (1967) found that the zygote obtained by fertilization of *Strongylocentrotus nudus* ova via *S. intermedius* spermatozoa showed the same level of heat tolerance as a normal *S. nudus* zygote, indicating the dominant influence of the eggs' thermal characteristics. Hinegardner (1975) crossed the sand dollars *Encope californicus* and *Dendraster excentricus* and reported that during accidental exposure of larvae to high temperatures, physiological responses to stress were inherent in the hybrids. Furthermore, Hinegardner found that the hybrids exhibited paternal characteristics, which is considered to be a general tendency of echinoderm hybrids. Lucas and Jones (1976) found that hybrids of the asteroids *Acanthaster planci* and *A. brevispinus* were morphologically intermediate. Marcus (1980) also found intermediate differences indicating joint influences of maternal and paternal genomes in the echinoid *Arbacia punctulata* from two geographic localities. Scheibling (pers. obs.) found that Type 2 hybrids reared in the laboratory morphologically resemble the maternal parent. Cytoplasmic constituents in the eggs may influence the developing larvae in *Echinaster*. The genetic influence of the maternal and paternal genomes is apparently variable in echinoderms.

The differences in the development and growth of the sibling species of *Echinaster* appear to be genetically controlled, and not the result of environmentally induced modifications of their physiology. Although most echinoderm hybrids do not live beyond the larval stage (Hinegardner, 1975), Scheibling (1982) reared Type 2 hybrids for one year in the laboratory and found their growth rates to either equal or exceed that of Type 2 wild individuals. The high degree of genetic compatability between the two species, as demonstrated by the $F_1$ hybrids, indicate that the two species are closely related and suggests recent speciation.

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# ABSTRACTS OF PAPERS PRESENTED AT THE GENERAL SCIENTIFIC MEETINGS OF THE MARINE BIOLOGICAL LABORATORY

AUGUST 17–20, 1982

*Abstracts are arranged alphabetically by first author within the following categories: actin, microtubules, membrane transport, and microanatomy; ecology; fertilization and development; neurobiology; parasitology and pathology; photoreceptors; and physiology and biophysics. Author and subject references will be found in the regular volume index in the December issue.*

## ACTIN, MICROTUBULES, MEMBRANE TRANSPORT, AND MICROANATOMY

### *Glutamate dehydrogenase activity in wood- and mud-burrowing bivalve molluscs.* CRAIG J. ANMUTH (Oberlin College), S. M. GALLAGER, R. MANN, AND R. S. ALBERTE.

Glutamate dehydrogenase (GDH) catalyzes the interconversion of $\alpha$-ketoglutarate and L-glutamate: $\alpha$-ketoglutarate + $NH_4^+$ + NADH or NADPH $\rightleftharpoons$ L-glutamate + $NAD^+$ or $NADP^+$ + $H_2O$. In animal tissue this enzyme is localized in mitochondria and uses either NADH or NADPH. The bacterial enzyme, however, is NADPH specific. We examined the energy substrate specificity and activity of GDH and ammonia uptake or excretion in the wood-boring bivalves (shipworms) *Lyrodus pedicellatus*, *Bankia gouldi*, and *Teredo navalis*, and the mud-burrowing clam *Solemya velum*. All these animals possess symbiotic bacteria (unpublished data).

Animals were maintained in ambient sea water (ammonia concentration 2 $\mu$mole). The shipworms were dissected into gill tissue, tissue encompassed by the valves, and remaining tissues, while *S. velum* was dissected into gill tissue and remaining tissue. The tissues were homogenized, subcellular components collected by differential centrifugation, and the pellets lysed to release GDH. The reaction was assayed in the forward direction by the oxidation of NADH or NADPH. Whole animal NADH-dependent GDH activities were 156.86 ± 13.0 (s.e.; N = 3), 123.81 ± 32.1 (N = 3), 49.43 (N = 1) 72.53 ± 13.5 (N = 3) m*M* NADH/g wet weight/h for *L. pedicellatus*, *B. gouldi*, *T. navalis*, and *S. velum*, respectively; while the NADPH-dependent activities were generally higher: 195.11 ± 52.1, 157.84 ± 69.9, 41.23, 122.46 ± 46.9 m*M* NADPH/g wet weight/h, respectively. Activity was greatest in the gill tissues. Prior long term incubation of animals at 50 $\mu$mole ammonia concentrations reduced GDH activity.

A significant portion of NADPH-dependent GDH activity could be bacterial in origin. High NADPH-specific GDH activity was found in a monoxenic culture of bacteria isolated from the gill tissue of *L. pedicellatus*. All shipworm species exhibited net ammonia rates of 1–2 $\mu$mole $NH_4^+$/g wet weight/h for intact animals at ambient ammonia concentrations. In contrast *S. velum* exhibited net ammonia excretion. It is possible that the symbiotic bacteria present can account for a significant portion of the ammonia uptake by *L. pedicellatus* and contribute to its nitrogen metabolism.

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### *A possible role of protein carboxymethylase in fertilization and sperm motility.* C. ARANOW, J. COHN, AND W. TROLL.

The evaluation of sperm motility is an important factor in determining the cause of infertility. Nonmotile or poorly motile sperm of many species can not successfully fertilize eggs. In man, low sperm motility has been shown to correlate with low fertility (Gagnon *et al.* 1982). We examined protein carboxymethylase (PCM), an enzyme described in a diversity of biologic systems such as hormone exocytosis, bacterial chemotaxis, and leukocyte stimulation and migration. Its activity has been observed in the membrane fractions of motile sperm. PCM catalyzes the methylation of free carboxyl groups of protein substrates to form methyl esters using S-adenosyl methionine (SAM) as a methyl donor. The methyl ester bond formed in this reaction is hydrolyzed either spontaneously at neutral or alkaline pH or enzymatically by a protein methylesterase releasing methanol. We assayed for PCM by incubating sea water with sperm and tritium-labeled methionine and determined the radioactive methanol extracted by toluene. The sperm incorporates methionine which reacts with ATP to form labeled SAM. Labeled methyl groups donated in the PCM reaction hydrolyze releasing radioactive methanol. Shaking the scintillation vial at selected times extracts the methanol generated by the system into a toluene scintillent which is counted. In motile sperm, an initial rapid accumulation of methanol counts released by the enzyme system plateaus with time. We examined the effect of $H_2O_2$ and dithiothreotol (DTT) on PCM since $H_2O_2$ has been implicated in the prevention of polyspermy (the penetration of the egg by more than one sperm) in *Arbacia punctulata*. $H_2O_2$ decreases both the fertility and motility of *Arbacia* sperm while DTT reverses these effects. We now report that $H_2O_2$ appears to inhibit while DTT potentiates the PCM enzymatic system. Thus the mechanism of $H_2O_2$ inactivation and DTT reactivation of *Arbacia* sperm may be due to the effect of these compounds on PCM.

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### *Fine structure of tissue warming the brain and eye in tuna.* BARBARA BLOCK (Duke University), EUGENE COPELAND, AND FRANK CAREY.

The gills of teleosts have a total surface area that is efficiently arranged to obtain gas exchanges by diffusion. The same physical structures provide very efficient heat transfer so that convection cooling keeps the general body temperature within a fraction of that of the ambient water. Some tissues, usually those associated with survival capabilities, have developed thermogenic properties. The released heat is conserved by vascular counter current heat exchanges. The recent discovery of thermogenic tissue associated with the brain of swordfish (Carey 1982, *Science* **216**: 1327–1329) caused us to examine a similarly located tissue in bluefin tuna where we have recorded as high as 13.2°C temperature elevation in the brain. A hot spot was located in pigmented tissue that incorporates a counter current vascular supply to both eye and brain. Grossly, the tissue was opalescent and fatty in appearance with punctate localizations of melanin. At the fine structure level the cells (other than the classical melanocytes) at first glance look like partially compacted leucocytes, both granular and agranular. The major population is granular and marked by having droplets in various stages that are only partially osmiophilic (*i.e.* largely saturated lipid?). The droplets are consistently associated with varying amounts of non-membrane bound granular material. Occasionally, a very large osmiophilic lipid droplet is seen. The much fewer agranular cells usually have a dense array of rough endoplasmic reticulum filled with material. None of the cells showed the dense concentration of mitochondria seen in the similarly located tissue of the swordfish. Since peroxisomes are known to catalyze metabolic reactions in such a way that heat is a byproduct, the tissues were checked histochemically with a diaminobenzidine technique. The preliminary results were interesting but not conclusive.

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### *Temperature-induced disassembly of isolated marginal bands and reassembly of marginal band tubulin.* WILLIAM D. COHEN (Hunter College, NY), GEORGE M. LANGFORD, AND ROGER D. SLOBODA.

The marginal bands (MBs) of microtubules in living dogfish erythrocytes are cold-labile, and reassemble upon rewarming. However, isolated dogfish erythrocyte MBs are stable at 0°C in a medium consisting of 1 m*M* $MgCl_2$, 5 m*M* EGTA, 100 m*M* PIPES, pH 6.8 (Cohen *et al.* 1982, *J. Cell Biol.* **93:** 828–838). Observations by Diana Bartelt (Ph.D. Dissertation, CUNY, 1982) suggested that this was due to insufficient ionic strength. Using darkfield microscopy in the present work, isolated MBs were observed to disassemble at 0°C in 30–60 minutes if 150 m*M* KCl was included in the medium. To test for tubulin reassembly, GTP was added to 1 m*M* and samples were rewarmed on slides. Rodlets appeared within a few minutes, and subsequently formed masses of fibrillar elements, some approaching MBs in thickness. Reassembly was inhibited by 1 m*M* colchicine. Formation of the fibrillar aggregates appeared to involve rodlet alignment resulting from flow, and the aggregates dispersed into individual rodlets after vigorous tapping on the coverslip. Further study will be required to determine whether the organization of the fibrillar aggregates resembles that of MBs. At 150 m*M* KCl, reassembly occurred at approximately 0.4 mg protein/ml, but not at 0.2 mg/ml. However, reassembly did occur at 0.2 mg/ml in 15 m*M* KCl, showing that lower salt concentrations favored assembly. Negative staining of reassembled material revealed principally normal-looking microtubules, some of which adhered to others along their length. After centrifugation of 0°C-disassembled MB preparations at $100{,}000 \times g$ for 30 min (approx. 4°C), reassembly occurred as before, demonstrating that it did not involve the presence of MB microtubule seeds. Following reassembly spectrophotometrically with such preparations, absorbance (350 or 400nm) rose to a plateau in a few min at 25°C. SDS-PAGE showed that the reassembled material consisted of tubulin plus smaller amounts of two low molecular weight proteins (LMWs). Studies are in progress to characterize further MB tubulin and to determine whether the LMWs are MB microtubule-associated proteins (MAPs).

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## *Lability of mitotic spindle microtubules during cell lysis.* L. CRESWELL, T. OTTER, D. A. LUTZ, AND S. INOUÉ (Marine Biological Laboratory).

The transition of mitotic spindles from their living state to a less labile *in vitro* or "isolated" condition was studied by lysing sand dollar (*Echinarachnius parma*) eggs within the fertilization envelope. When eggs are lysed in PEM buffer (10 m$M$ PIPES, pH 6.8, 5 m$M$ EGTA, 0.5 m$M$ $MgCl_2$), the birefringence (BR) of the half-spindle decays to 5% of its initial value within 10 minutes. When Triton X-100 (0.1% or 1%) is included in the PEM, the initial rate of BR decay slows, and the final BR is stabilized at 20% to 40% of the initial value. During extraction in PEM plus Triton, the cytoplasm disperses and the spindle appears more fibrous. Spindle BR disappears upon cooling (5°C for 10 minutes) two minutes after lysis, but not upon cooling eight minutes later. To our surprise, when either Colcemid (CLM, 10 or 20 $\mu M$) or colchicine (100 $\mu M$) was included at lysis, the spindle BR did not decay at all for 30 minutes. Spindles were stable (BR 110–140% of initial value) in lysis medium plus CLM with or without detergent. To test whether this apparent stabilization was due to CLM itself or an unknown contaminant, we replaced CLM in the lysis buffer with its inactive analog lumicolcemid (LCLM), prepared by irradiating the same CLM solution with 366 nm light. When spindles are isolated with buffers containing LCLM (20 $\mu M$), the BR decay curve closely resembles the curve for spindles isolated in PEM buffer alone. Because the LCLM solution should contain the same contaminants as the CLM solution, it appears unlikely that the stabilization observed in CLM is due to an unknown contaminant. While 20 $\mu M$ CLM stabilizes the isolated spindle for over 30 minutes, this same dose of CLM induces complete loss of spindle BR in 8 minutes in the living cell. Therefore, the isolated spindle is no longer CLM-labile, it is stabilized instantly by exposure to PEM buffer containing CLM, and it loses cold lability in a few minutes.

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## *Dissociation constants of dimeric actin cross-linking proteins.* GEORGE Q. DALEY AND NORMA ALLEWELL (Wesleyan University, CT).

A number of structurally similar reversibly associating proteins purified from cytoplasmic extracts cross-link actin filaments to form isotropic gels. The cross-linking efficiency of these proteins depends upon not only their affinity for actin but also the extent to which they self-associate. Determination of the equilibrium constants for self-association and actin-binding permit quantitative comparisons of the ability of these proteins to contribute to the gelation of cytoplasmic actin.

Both values were measured for the dimeric actin cross-linking protein filamin. Filamin was purified from chicken gizzard by the method of Feramisco and Burridge (1980, *J. Biol. Chem.* **255**: 1194–1199). The binding affinity ($K_D$) of filamin for F-actin was determined by a co-sedimentation assay. F-actin, (2.4 $\mu M$), was mixed with various concentrations of filamin in 0.1 $M$ KCl, 1 m$M$ Ca/EGTA (R = 0.05), 1 m$M$ Mg-ATP, 20 m$M$ PIPES, pH 6.8 (volume 0.2 ml) and incubated for 30 min at 25°C. F-actin and filamin alone served as controls. The solutions were centrifuged at 30 PSI (100,000 × *g*) for 30 min at RT in an air-driven microcentrifuge. Virtually all of the actin (90%) but none of the filamin (4%) in the controls pelleted under these conditions. Filamin which sedimented in the presence of F-actin was therefore assumed to be bound. To determine the quantities of actin and filamin 10% SDS-polyacrylamide gels of the pellets and supernatants stained with coomassie blue R were scanned densitometrically. Scatchard analysis of the binding data yielded a $K_D$ of $2.1 \times 10^{-7}$ $M$ and a binding ratio of 1 filamin dimer for every 13 actin monomers.

The $K_D$ for the monomer-dimer equilibrium was determined by analytical gel chromatography (Valdes & Ackers 1978, *Meth. Enz.* **61**: 125–142) using high pressure liquid chromatography (HPLC TSK-400 column, Biorad). From the measured $K_D$ value of $2 \times 10^{-6}$ $M$ a free energy of association for the filamin dimer was calculated to be −7.7 kcal/mole.

Supported by NIH Training Grant GM-31136-04. HPLC equipment was generously loaned by Biorad.

### *Isolation and study of metaphase and anaphase meiotic spindles from* Chaetopterus *oocytes.* Dennis Goode and Vidya Sarma (University of Maryland, College Park).

We have developed methods to isolate and study the meiotic spindles from *Chaetopterus* oocytes, which enter and become naturally arrested in first meiotic metaphase shortly after exposure to natural sea water. Since oocytes are much larger than the spindles and contain birefringent yolk granules, a useful first step is to prepare "mini-cells." Metaphase eggs resuspended in calcium-free sea water are placed over a 1 $M$ sucrose solution containing 10% calcium-free sea water and centrifuged at 25°C and 24,000 $\times g$ for 10 min. The cells removed from the interface between the solutions are primarily small, clear cells containing meiotic spindles. These spindles remain arrested in metaphase and can be increased or decreased in size by temperature shifts or mitotic inhibitors. For example, exogenous maytansine (2.5 $\mu M$) produces spindle disassembly in 3 min. Anaphase mini-cells are prepared by adding sperm 1 min before centrifuging fertilized eggs on a sucrose cushion for 5 min. When mini-cells are lysed with Nonidet P-40 in microtubule assembly buffer plus 2 mg/ml bovine brain tubulin and $10^{-3}$ $M$ GTP, the birefringence of the spindles is enhanced. When lysed in the same medium containing 2 mg/ml dichlorotriazinylaminofluorescein-labeled microtubule proteins and incubated for 12 min at 32°C, the entire spindle becomes fluorescent; but when incubated for 5 min, fluorescence is concentrated in two regions just poleward from the metaphase chromosomes. Since clean spindles are difficult to isolate from oocytes, we use mini-cells as an intermediate step in isolation. Mini-cells are washed in isolation medium without detergent, then pelleted and resuspended in 100 times their volume of isolation medium (2 $M$ glycerol, 50 m$M$ PIPES, 5 m$M$ EGTA, 0.5 m$M$ $MgCl_2$, and 0.5% Nonidet P-40 at pH 6.8) until cells lyse. Anaphase meiotic spindles can be isolated from anaphase mini-cells for analysis and comparison with metaphase spindles.

Supported by NSF grant PCM-8011474.

### *Regions of microtubule assembly in isolated spindles of* Spisula solidissima. Leah T. Haimo (University of California, Riverside) and Bruce R. Telzer.

Microtubules (MTs) may be described by two parameters, their structural polarity and their polarity of assembly. MTs within a half spindle have been shown previously to be oriented with their plus ends located at the equatorial plate. Studies were now undertaken to determine the polarity of assembly of these MTs. Spindles were isolated from oocytes of *Spisula solidissima* and then incubated with *Tetrahymena* or *Chlamydomonas* dynein which resulted in the uniform decoration of all spindle MTs. These dynein-decorated spindles were then incubated in 6S tubulin which was incorporated into the spindles as indicated by an increase in their birefringence and in size of asters. Gel electrophoresis revealed that both dynein and 6S tubulin cosedimented with the spindles. Electron microscopy was undertaken to distinguish between dynein-decorated, native MTs and undecorated, newly assembled MTs. Undecorated MTs were observed surrounding the periphery of the spindle. In addition, within the spindle the number and length of undecorated MTs occurred with increasing frequency nearer the equator. That native spindle MTs did, in fact, elongate during the incubation in tubulin was suggested by the observation that some MTs possessed two domains, one, closer to the pole, dynein decorated, and the other, distal to the pole, undecorated. These domains represent native and newly assembled regions of MTs, respectively. In summary, new MT assembly occurred primarily around the periphery and in the equatorial region of isolated spindles. Experiments are currently underway to determine the polarity of assembly of the kinetochore MTs.

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### *Further studies on the ultrastructure and distribution of lateral line and ocular-associated structures (possibly sensory) in a marine teleost* (Stenotomus chrysops). Clifford V. Harding, Stanley R. Susan, Woo-Kuen Lo, S. Gregory Smith, and Vinay Reddy (Kresge Eye Institute, Wayne State University, Detroit, MI).

Epidermal projections near the eye and lateral line system have been observed in the scup. Each projection consists of four cell types: a single core cell, a monolayer of epithelium, modified epithelial cells at the basal region (collar cells) where the projection bends passively in response to water movements, and the bulb cell, which has a complex and unique structure. The distal end of the bulb cell has a prominent bulb, connected by a long cytoplasmic stem to the cell body located in the epidermis proper. The stems are completely enveloped by epithelial cells, each of which envelops a portion of the stem, and shows extensive desmosomal connections where one portion of the cell meets the other portion of the same cell (like a collar button). These epidermal cells give the impression of primitive Schwann cells. The bulb cells (which number 6–8 and run parallel to the core cell) are also unique among the four cell types in not having *any* desmosomal connections. The overall arrangement of the cells within the projections suggests a sensory function, perhaps the detection of the direction of water movement (with the bulb cells serving as differential detectors of the direction of projection bending). As yet, however, we have no definitive evidence for nerve connections with any portion of the projection. Studies on distribution show that the number of projections per scale is several-fold higher dorsal to the lateral line as compared to ventral regions. The single row of specialized scales along the lateral line form a distinct line of demarcation between the dorsal scales with large numbers of projections and the ventral scales with low numbers. Further studies of distribution may provide additional clues about the function of these small but complex structures.

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### *Some membrane structural changes accompanying morphogenetic changes in* Tetrahymena. LINDA A. HUFNAGEL (University of Rhode Island).

The surface of the ciliated protozoan cell is covered by three membrane layers, the plasma membrane (PM) and the inner and outer membranes of numerous cortical alveoli which directly underlie the PM. How these membranes are assembled is not understood in spite of an intimate role in cortical morphogenesis. Therefore, freeze-fracture EM has been used to analyze membrane changes accompanying two developmental sequences in the ciliate *Tetrahymena thermophila.* These sequences are 1) tip transformation, a remodeling of the anterior tip of the cell preceding pairing during conjugation, and 2) rapid cell growth induced by refeeding of cells starved overnight. During tip transformation, new linear assemblies of intramembranous particles (IMPs) develop within the protoplasmic face (PF) of the PM, marking the future boundary between remodeled regions of the cell cortex and remaining unaltered cortical regions. Oriented PF arrays normally found anterior to these new assemblies then disappear and the PF in this region becomes relatively devoid of IMPs. Also in tip transforming cells, rosettes of particles, hitherto undescribed in *Tetrahymena*, appear on the PF of the membranes covering anteriorly situated cilia. These rosettes lie close to the ciliary plaques. Their random positions suggest that they may be free to move laterally within the membrane. While the functions of these arrays are unknown, it seems likely that linear assemblies are important in shape changes that precede cell pairing while ciliary rosettes are related to recognition between mating types. In starved-refed cells sampled about 4½ to 5½ h following refeeding, two different types of smooth membrane vesicles were associated with the alveolar membranes and the PM. In addition, periodic blebbing of the membranes was observed in refed cells but not starved controls. These results suggest several routes for introduction of constituents into cortical membranes of rapidly growing cells.

### *Evidence for the association of high molecular weight proteins (MAP 2) with a subset of microtubules* in vitro. GEORGE M. LANGFORD (University of North Carolina, Chapel Hill) AND ADRIAN C. LAWRENCE.

Recent studies have shown that two populations of microtubules can be identified in intact neurons by immunofluorescence microscopy. We designed experiments to test whether two populations of microtubules can be reconstituted *in vitro* from purified brain microtubule proteins (MTPs). Our results show that two populations of microtubules can be identified in samples of microtubules reconstituted *in vitro.* These findings support the hypothesis that purified brain MTPs self-assemble with sufficient molecular specificity to preserve the molecular identity of the subsets of microtubules which existed in the cells. For these experiments, we purified MTPs from the brain of cattle, shark, and squid. When analyzed by SDS-PAGE, the bovine brain MTP samples contained two types of microtubule-associated proteins (MAPs), the high molecular weight (HMW) and tau proteins. Shark and squid MTPs contained two types of MAPs, but the HMW MAPs of these two organisms were different from those of cattle. When assembled, all three types of MTPs showed two populations of microtubules. The two populations were identified by negative contrast electron microscopy. One type of microtubule, representing the majority population, appeared as smooth, straight, randomly dispersed filaments. The other type appeared as wavy filaments that were always cross-linked into large tangled bundles. The basis of cross-linking was found to be due to the presence of lateral projections that decorated the surface of these microtubules. The lateral projections fit the description of HMW MAP 2. The smooth and decorated microtubules had similar rates of depolymerization upon dilution. We hypothesize that the two types of microtubules arise by the simultaneous formation of two kinds of nuclei, one kind containing tau and the other HMW MAPs. The nuclei may form by the cooperative interactions between a given class of MAPs and tubulin. The ratio of the two kinds of nuclei probably depends upon the ratio of the two kinds of MAPs. The two forms of microtubules are thought to represent the axonal and dendritic populations of microtubules in neurons.

(A.C. Lawrence was a student in the Rockefeller Foundation Program in Life Sciences for High School Students.)

### *Zonulae occludentes and transepithelial permeability in the ocular lens epithelium.* Woo-Kuen Lo and Clifford V. Harding (Kresge Eye Institute, Wayne State University, Detroit, MI).

The existence of zonulae occludentes (tight junctions) in ocular lens epithelium is uncertain. By utilizing a "double mounting" method in freeze-fracture electron microscopy, we have demonstrated the presence of zonulae occludentes structures in the lens epithelia of human and frog (*Rana pipiens*) for the first time. It was found that these zonulae occludentes are always located between the lateral membranes of epithelial cells in close proximity to the apical end of the cells. The zonulae occludentes are characterized by a number of continuous anastomosing grooves or strands on the E-face of the membrane.

The transepithelial barrier function of zonulae occludentes in the lens epithelia of frog and sea bass is determined by a "wash out" procedure, in comparison with the conventional "non-wash" procedure for the protein tracer HRP (horseradish peroxidase). In the "wash out" experiments, frog lenses were washed in tissue culture medium TC-199 (70%) for various periods of time (2, 7.5, 10, 15, and 20 minutes) immediately following 15 minutes of enzyme incubation within the eye (1% HRP in TC-199 was injected into the anterior chamber). The lenses were then fixed and processed for cytochemistry. We have found that within various time intervals of washing, HRP reaction product is consistently blocked at the location of membrane fusions (zonulae occludentes), as seen with thin-section transmission electron microscopy. This corresponds to the location of zonulae occludentes found in the freeze-fracture studies. By applying the same "wash out" procedure to the sea bass lenses, we have obtained results similar to those found in the frog lens. Thus, these data strongly suggest that there are zonulae occludentes in the lens epithelia of human, frog, and sea bass, and that these structures do provide a barrier function for the transepithelial diffusion of HRP (molecular weight 40,000 daltons).

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### *L-leucine transport by isolated toadfish hepatocytes.* Roger Persell and Audrey E. V. Haschemeyer (Hunter College).

Hepatocytes were isolated from 20 ± 1°C-acclimated toadfish (350–400 g) by liver perfusion *in situ* with a $Ca^{++}$-free buffered balanced salt medium, followed by a medium containing 1.7 m$M$ $CaCl_2$, 100 units/ml collagenase Type IV (Sigma), and physiological concentrations of 19 amino acids (excluding leucine). Cells were collected by combing, washed twice with collagenase-free medium, and resuspended at 0.1 g/ml in 240 m$M$ NaCl, 5 m$M$ KCl, 1.7 m$M$ $CaCl_2$, 0.4 m$M$ $KH_2PO_4$, 0.3 m$M$ $Na_2HPO_4$, 4 m$M$ Na $HCO_3$, 5.6 m$M$ glucose, 10 m$M$ HEPES (pH 7.8), and 1% bovine serum albumin. Transport experiments were carried out in Beckman microfuge tubes by addition of 100 $\mu$l cell suspension to 100 $\mu$l medium containing 0.1 $\mu$Ci L-$^{14}$C-leucine, 0.2 $\mu$Ci$^3$H-inulin and additions as indicated below. Reaction was terminated after times of 5 s to 3 min by centrifugation, removal of the supernatant, and rinsing of the cell pellet. Intracellular uptake of $^{14}$C-leucine was determined after correction for extracellular radioactivity by use of $^3$H recovery. Uptake at 0.1 m$M$ external leucine concentration followed first-order kinetics ($k = 1.1 \pm 0.1$ min$^{-1}$ at 21°) to a plateau at $t = 2$ min, corresponding to a space occupied of 1–2 $\mu$l per cell pellet, after which a continuous slow linear uptake occurred. Concentration dependency of the early time course corresponded to Michaelis-Menten kinetics with $K_m = 0.52$ m$M$ and $V_{max} = 760$ pmole/$\mu$l · min. The principal inhibiting amino acids (tested at 2 m$M$) were isoleucine and phenylalanine; smaller effects were noted with valine and methionine.

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### *Uptake and utilization of L-alanine by 10 species of bivalve molluscs.* Robert D. Prusch, Scott M. Gallager, and Roger Mann (Woods Hole Oceanographic Institution).

The influx of dissolved L-alanine into isolated gill and mantle tissue of adult specimens of 10 species of bivalves was examined. Isolated tissue was incubated for 2 h in 10 ml of sea water containing 20 $\mu M$ L-alanine ($^{14}$C) while the time course of L-alanine depletion from the medium was followed.

Influx was consistently higher in isolated gill rather than isolated mantle. Influx rates for isolated gill were, in decreasing order: 40, 27, 16, 11, 9, 8, 6, 4, 3, 2 nmol/mg dry wt/h for *Lyrodus pedicellatus, Argopecten irradians, Solemya velum, Modiolus modiolus, Crassostrea virginica, Mya arenaria, Bankia gouldi, Mytilus edulis, Teredo navalis,* and *Mercenaria mercenaria,* respectively. Additions of 0.5 m*M* cyanide terminated influx in all cases indicating that flux was effected by active transport. Although the three species of shipworms examined (*L. pedicellatus, B. gouldi,* and *T. navalis*) possess symbiotic nitrogen-fixing bacteria in their gills (Waterbury and Calloway, in prep.) a consistently higher influx of amino acid was not evident in the shipworms when compared with the other species examined. Uptake of L-alanine by isolated gill of *L. pedicellatus* followed Michaelis-Menten kinetics with a $V_{max}$ of 0.086 $\mu$mol/mg dry wt/h and a $K_m$ of about 20 $\mu M$ within a range of ambient L-alanine concentrations of 1–200 $\mu M$.

Fractionation of adult *B. gouldi* following both 20 and 90 hour incubation periods with 20 $\mu M$ L-alanine showed distribution of the $^{14}C$ label throughout the protein, carbohydrate, and lipid fractions. Pediveliger larvae of *B. gouldi* exhibited a $V_{max}$ of 0.015 $\mu$mol/mg dry wt/h and a $K_m$ of about 14 $\mu M$. After a one hour incubation in 1 $\mu M$ L-alanine about 60% of the $^{14}C$ label was accounted for in larval tissue and about 20% was collected as respired $^{14}CO_2$.

L-alanine accumulated from the sea water is, therefore, actively metabolized by both larval and adult shipworms and may account for a major portion of their nitrogenous nutrition.

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### *Rapid rates of colchicine- or colcemid-induced spindle microtubule disassembly* in vivo*: implications for the mechanism of microtubule assembly.* E. D. SALMON, M. MCKEEL, T. S. HAYS, AND C. RIEDER (Dept. of Biology, University of North Carolina, Chapel Hill).

Following perfusion of metaphase and anaphase cells with culture media containing 10 m*M* colcemid, spindle birefringent retardation (BR) decreased rapidly to 10 percent of its initial value within a characteristic period, T, of 30–60 sec for first mitotic spindles of *Lytechinus variegatus* (24°C), first meiotic spindles of *Chaetopterus pergamentaceus* (24°C), and for $Ptk_1$ tissue culture cells (34°C). BR was measured in the central half-spindle region by a calibrated voltage from a video "spot meter." Elimination of plasma membrane permeability effects was achieved by the microinjection of colchicine or colcemid into early division *Lytechinus* cells. For intracellular colchicine concentrations of 0.1–5 m*M*, $T = 15–25$ sec, independent of concentration. Below 0.1 m*M*, T depended inversely on the concentrations of colchicine or colcemid. Ultrastructural analysis showed that the rapid loss of spindle BR was due to depolymerization of non-kinetochore fiber MTs; kinetochore fiber MTs were differentially stable. Lumicolchicine at 0.5 m*M* intracellular concentrations had no effect on spindle BR or cell division. If colchicine and colcemid block only the assembly reactions by binding to subunits in the spindle tubulin pool, then the intrinsic rate of dissociation of tubulin from non-kinetchore MTs is 600 dimers/sec for an average initial MT length of 7.5 $\mu$m and $T = 20$ sec. This rate is about 50-fold greater than reported rates of dimer association to a single end of a MT *in vitro* at a tubulin critical concentration of 2 $\mu$M. Consequently, subunit exchange may occur at multiple sites along spindle MTs *in vivo*.

Supported by NIH grant 24364.

### *Interactions of several heavy metals with L-leucine transport in the intestine of the toadfish,* Opsanus tau. R. SOCCI, N. CURTIS, A. FARMANFARMAIAN (Rutgers University), AND A. ZWEIFACH.

It is known that heavy metals inhibit enzymes and transport systems in various mammalian tissues. We have examined the effect of $HgCl_2$, $CH_3HgCl$, $CdCl_2$, and $SrCl_2$ on the intestinal absorption of 0.25 m*M* L-leucine by the toadfish, *Opsanus tau*.

The absorption of $^{14}C$-labeled L-leucine from buffered fish Ringer's *in vitro* was measured in the presence and absence of a heavy metal. $^3H$-inulin was used as a water marker. $Hg^{2+}$ produced significant ($P < 0.01$) inhibition of uptake at all concentrations tested—20% at 2.5 ppm, 40% at 5 ppm, 54% at 10 ppm, and 67% at 20 ppm. $CH_3Hg^+$ inhibited uptake significantly ($P < 0.05$) by 22% at 5 ppm, 30% at 10 ppm, and 46% at 20 ppm, but not at 2.5 ppm. Neither $Cd^{2+}$ nor $Sr^{2+}$ produced significant inhibition in the time-concentration range tested (2.5–20 ppm at 10 min). Preliminary experiments showed that the removal of $Ca^{2+}$ or $Mg^{2+}$ from the Ringer's caused a moderate level of inhibition in leucine transport, this inhibition was not synergistic with that of $Hg^{2+}$ when the latter was added at 10 ppm.

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### *Vigorous movement of sand dollar sperm during extraction with Triton X-100.* CYNTHIA L. SUNDELL AND TIM OTTER (Marine Biological Laboratory).

Sand dollar (*Echinarachnius parma*) sperm treated with Triton X-100 become hyperactive for about ten seconds, then stop until they are reactivated by addition of ATP. We studied this brief transition ("the burst") from living sperm to "Triton-model" using frame-by-frame motion analysis of video recordings. Living sperm near a glass surface normally swim in circular or open spiral paths of characteristic diameter. Flagellar movement is nearly symmetric. Occasionally, the sperm stop in cane-shaped configurations for one or two seconds before resuming normal motility (similar to behavior described by Gibbons 1980, *J. Cell Biol.* **84:** 1–12). During the "burst" induced by addition of detergent, flagellar beating is highly asymmetric. Many sperm appear C-shaped, with a large principle bend and little or no compensating reverse bend. Since the flagellum is curved to one side, the sperm swim in a tight spiral path. Finally they thrash about in a frenzied pitching motion. Neither behavior was seen in untreated sperm. At the end of the burst, the sperm stop abruptly in configurations resembling "rigor waves." Many sperm have two round swellings at the midpiece. After extraction (with 0.15 *M* KCl, 2 m*M* $MgSO_4$, 2 m*M* Tris-HCl, 5 m*M* $CaCl_2$, 1 m*M* EDTA, 0.04% Triton X-100, pH 8.2), ATP-reactivated sperm beat with nearly symmetric waveforms and swim in straight lines. Thus, the asymmetric beating seen during the burst is not due to a permanent structural change in the flagellum upon extraction. To test whether a release of mitochondrial ATP causes the burst, we immobilized sperm with DNP (1 m*M* in filtered sea water) prior to extraction and reactivation. Surprisingly, DNP-immobilized sperm also repond with a typical burst. Therefore, treating sperm with the extraction buffer causes a burst in activity that is not due to a sudden release of mitochondrial ATP, and that briefly restores motility to DNP-immobilized sperm.

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## ECOLOGY

### *Effects of eutrophication on the increase of chlorophyll* a *in phytoplankton from coastal waters.* OSIRIS BOUTROS (University of Pittsburgh at Bradford, Bradford PA 16701), NINA CARACO, WILLIAM DENNISON, AND IVAN VALIELA.

Phytoplankton nutrient enrichment experiments were performed in sea water (Vineyard Sound, 32ppt salinity) and brackish water (Salt Pond, 28 ppt salinity; Siders Pond, 4 ppt salinity), all from the Falmouth, MA area.

The effects of enrichment were assessed by fluorometric and spectrophotometric determination of chlorophyll *a*.

Combined additions of nitrogen and phosphorus produced the greatest increases in growth in all three waters with the greatest increases recorded in Salt Pond water. Phosphorus increased growth in Siders Pond water almost as much as the combined addition of nitrogen and phosphate but had little or no effect in Vineyard Sound and Salt Pond waters. Nitrogen addition produced a greater response in Vineyard Sound water than in Salt Pond and had no effect on growth in Siders Pond.

Phosphorus was limiting in the low salinity water (Siders Pond), and nitrogen was limiting in higher salinity waters (Salt Pond and Vineyard Sound).

### *Frequency of resistance to selected antibiotics and heavy metals and the occurrence of plasmids in enteric bacteria from a marine source.* SUSAN BOUTROS (University of Pittsburgh at Bradford, Bradford, PA 16701), WILLIAM REZNIKOFF, JEFF GARDNER, AND NANCY V. HAMLETT.

A previous study of resistance in enteric bacteria from two marine ponds in the Woods Hole area found high levels of resistance to kanamycin, ampicillin, and mercury (Javero 1981, unpublished). The present study was done to determine the current levels of resistant bacteria in the same marine ponds and whether these bacteria possess plasmids.

Bacteria were collected from water samples from Eel Pond, Great Pond, and Salt Pond by filtration through nitrocellulose membranes (0.2 $\mu$m pore size). Enteric bacteria were selected and the proportions of salt-tolerant, metal-resistant, and antibiotic-resistant bacteria determined by placing the filters on MacConkey agar, MacConkey agar with 2% NaCl (Mac 2); Mac 2 + ampicillin (100 $\mu$g/ml); Mac 2 + kanamycin (20 $\mu$g/ml); Mac 2 + tetracyclin (15 $\mu$g/ml); Mac 2 + CdCl (100 $\mu$g/ml); Mac 2 + $PbCl_2$ (500 $\mu$g/ml); and Mac 2 + $HgCl_2$ (10 m*M*).

Approximately 10% of the enteric bacteria were resistant to antibiotics and/or metals. Of 75 resistant strains selected for further study, 55% were not salt dependent (non-marine), and 45% were salt requiring; 25% were resistant to one antibiotic or metal and 75% were resistant to two or more. The most common pattern combined resistance to one antibiotic with resistance to one or both metals. No Hg resistance was found. Resistance to two antibiotics was found only in non-marine forms.

Twenty-seven resistant strains were screened for plasmids using the technique of Kado and Liu (1981, *J. Bact.* **145:** 1365–1373). Plasmids were detected in 60% of these strains. Resistance to Cd and kanamycin was not consistently associated with plasmids. Resistance to tetracyclin and ampicillin was apparently associated with small plasmids, but additional work is needed to demonstrate that resistance is carried on transmissible plasmids.

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### *Seasonal variation in the flux of algal pigments to a deep-water site in the Panama Basin.* JONATHAN J. COLE (Ecosystems, Marine Biological Laboratory), SUSUMU HONJO, AND NINA M. CARACO.

A moored array of time-series sediment traps was deployed for an entire year at a station in the Panama Basin (3680 m deep; 5°17′N, 81°56′W) with traps set at 890, 2590, and 3560 m. At each depth, a six-membered rosette of collection chambers rotated beneath a large cone (1.5 $m^2$ diameter) such that each chamber collected the sediment which fell into the cone during a 60-day exposure. The chamber was automatically sealed and poisoned after the exposure.

At each depth the fluxes of algal pigments, organic carbon, and carbonate varied seasonally. Although most of the carbonate and much of the organic carbon was associated with the sinking of coccolithophorids, the flux of algal pigments was not. At 3560 m the peak flux of phaeopigment occurred in February–March; the peak flux of carbonate occurred in June–July.

Averaged over the year there was no difference in the quantity of algal pigments which arrived at the three depths. Although the lowest ratio of organic carbon:phaeopigment occurred at 890 m (40:1;mg:mg) and the highest ratios at 3560 m (285:1), on the average there was no trend with depth. These results suggest that decomposition in the water column below 890 m is slow. The quantity of phaeopigment arriving at the sea floor in the Panama Basin (80–280 $\mu g \cdot m^{-2} \cdot day^{-1}$) is roughly 50 to 100 times greater than the amount sinking to 2000 m at the Tongue of the Ocean and 10 to 70 times less than the amount sinking at the Peru Upwelling.

### *The effects of oil contaminated sediments on the growth of eelgrass* (Zostera marina *L.*). J. E. COSTA (Boston University Marine Program, Marine Biological Laboratory).

Eelgrass (*Zostera marina* L.) is widespread and locally important in temperate and cold water coastal ecosystems, but little research has been done on how hydrocarbon contamination of sediments affects eelgrass. No. 2 fuel oil recently impacted Buzzards Bay, MA, and in many sites 2 mg hydrocarbons/g sediment wet wt persisted for several years.

Sediment was collected at an unpolluted intertidal site near Naushon Island, MA, sieved to remove macrofauna, and contaminated with two No. 2 fuel oils: American Petroleum Institute (API) reference III, and Baytown, Texas Exxon (BTE) refinery oil. Before and after the experiments, eelgrass seedlings were weighed, and the rhizomes and leaves measured. A hole was punched in the leaf sheath; leaf production was measured by the outgrowth of scars. There were 7 treatments: 0 and 3.0 mg API oil/g sediment wet wt, and 0, 0.2, 1.0, 2.1, 6.2 mg BTE oil/g sediment wet wt. Seedlings were planted in an outdoor raceway system 12 days after sediments were oiled and immersed, and harvested three weeks later.

Eelgrass seedlings responded similarly to both oils. Biomass and leaf production (measured as % weight change, rhizome elongation, and relative leaf production), showed a linear decrease when plotted against log of oil concentration. At 0.2 mg oil/g sediment, leaf production and weight increase were 16% and 40%, respectively, below the control. Above 1.0 mg oil/g there was actual weight loss, 50% less leaf production, and inhibition of root and rhizome growth. Under field conditions wave action could uproot these plants. Above 2.1 mg oil/g, rhizomes often deteriorated, leaves were shed, and many plants senesced. In the API oil experiment chlorophyll *a* concentration decreased 60% providing an indication of the physiological effects that occurred. These results show that contamination of sediments with oil could have dramatic effects on the abundance and distribution of eelgrass.

Thanks to J. Capuzzo, B. Dennison, and I. Valiela for their assistance and advice. S. K. Alexander (Texas A & M) supplied the BTE oil. This work was in part funded by Sigma Xi.

## *Role of daily light period and intensity in photosynthesis and production of* Zostera marina *L. (eelgrass).* W. C. Dennison and R. S. Alberte (The University of Chicago).

Photosynthesis and growth responses of *Zostera marina* L. (eelgrass) are important in the adaptation of eelgrass to the nearshore marine environment. The influence of light regime on photosynthesis and production of *Z. marina* was examined with *in situ* manipulations of daily light periods and intensities. Underwater lamps and light shading screens were placed at shallow (1.3 m) and deep (5.5 m) stations in an eelgrass bed adjacent to the Fisheries Jetty in Great Harbor, Woods Hole, MA. Underwater lamps ($300\ \mu E \cdot m^{-2} \cdot s^{-1}$) extended the daily light period by 4 to 6 hours and shade screens (−60 to −80% of ambient light) shortened the daily light period by 3 to 5 hours for 30 days (June, 1982).

Leaf production rates at the deep ($5.1$ dry $g \cdot m^{-2} \cdot day^{-1}$) and shallow station ($3.6$ dry $g \cdot m^{-2} \cdot day^{-1}$) were reduced by 51 and 56%, respectively, in the shading experiments. Underwater lamps increased production by 15% at the deep station but had no effect at the shallow station. Photosynthesis versus irradiance (P vs I) curves for eelgrass indicate light compensation at $10\ \mu E \cdot m^{-2} \cdot s^{-1}$ with light saturation at $50\ \mu E \cdot m^{-2} \cdot s^{-1}$. P vs I curves were similar in shallow station experiments but variable in deep station experiments. Photosynthetic unit (PSU-$O_2$) size was doubled by shading at both stations. PSU density was reduced by 50% at the deep station shade but unaffected by the shallow station shade. An increase (92%) in leaf chlorophyll content occurred only at the shallow station shade with no changes in chlorophyll amounts or *a*/*b* ratios evident in other manipulations.

These results indicate 1) there is potential for molecular, cellular, and whole plant level adjustments by *Z. marina* to changes in light regime, 2) changes in daily light period play an important role in eelgrass production whereas the photosynthetic apparatus is affected by light intensity, and 3) different response mechanisms operate in plants growing at shallow and deep areas of the eelgrass bed.

Research supported by NSF Grant PCM-7810535.

## *Preliminary investigation of water quality and animal mortality at MBL's Department of Marine Resources.* Julie Early (Marine Biological Laboratory) and John Valois.

The effect of seasonal changes in the MBL seawater system on marine specimens kept in holding tanks is not known. MBL sea water has never been monitored for its chemical or physical changes. Without this information, the Laboratory for Marine Animal Health, under the direction of Dr. Louis Leibovitz, can not obtain a clear picture of what stresses are placed on animal stocks in holding tanks.

An initial examination of mortality and water quality is presented. Mortality and morbidity are monitored daily while water quality tests are completed biweekly. Water samples are collected from the intake and discharge of each of the buildings housing Marine Resource's animals. Samples are obtained from tanks demonstrating high mortality rates, as well. The water quality parameters tested include: pH, salinity, temperature, turbidity, ammonia, nitrite, nitrate, phosphate, sulfide, copper, and iron.

Values obtained at this point are as follows: pH, 7.6–8.2; salinity, 30–32‰; ammonia, 0.1–0.5 mg/l; nitrite, 0–0.05 mg/l; nitrate, 0–0.4 mg/l; phosphate, 0.004–0.2 mg/l; sulfide, 0–0.005 mg/l; copper, 0–0.02 mg/l; iron, 0 mg/l; turbidity, 0 FTU.

*Arbacia punctulata, Strongylocentrotus droebachiensis, Asterias forbesi,* and *Opsanus tau* had high mortality rates in the winter due to the extremely low water temperatures and bacterial attack. *Carcinus maenas, Cancer borealis,* and *Pagurus pollicaris* had high mortality rates in the winter and spring due to starvation and cannibalism. *Raja erinacea* suffered in the summer from circulatory disorders caused by the difference of the gas pressures in the water in which they were caught from that into which they were placed at MBL.

Contributing factors to mortality which influence water quality are the size and shape of the tank, the rate of water flow, the number of individuals per tank, the condition of the animals before being placed in the tank, amount of food added, and weather conditions (air temperature, % cloud cover, and precipitation).

For the future, it can be assumed that seasonal changes in both the chemistry and microorganisms in MBL sea water will be known. Some of these changes will be traced to mortality in specimens. This information will be useful in the construction of new seawater systems, and give good evidence as to when recirculating water might be most beneficial.

## *Nutrient flux and growth of the red alga* Gracilaria tikvahiae *McLachlan (Rhodophyceae).* Rodney M. Fujita (Boston University Marine Program, Marine Biological Laboratory).

Nutrient concentration and water flow rate determine the availability of nutrients to macroalgae. Increasing flow rate simultaneously breaks down diffusion gradients near the thallus and increases the rate of nutrient delivery. Thus, in the absence of diffusion gradients and under nitrogen-limited conditions, nitrogen flux (flow rate × concentration) should control growth rate.

*Gracilaria tikvahiae* was grown outdoors in 14-l flow-through tanks. The cultures were vigorously aerated to minimize diffusion gradients. *Gracilaria* required approximately 2 weeks to acclimate to the experimental conditions. At each of 3 flow rates (5, 10, and 20 culture volumes/day) replicate tanks were enriched to 3 different $NH_3$-N concentrations (0.05, 0.10, and 0.20 m*M*). The N:P ratio was maintained at 10:1. This resulted in 5 different nitrogen flux treatments (3.5, 7.0, 14.0, 28.0, and 56.0 millimoles N/day). Specific growth rate (SGR) was monitored as blotted wet weight increase. SGR increased as a linear function of nitrogen flux ($r = 0.79$; $SGR = 0.46$ (N-flux) $+ 4.9$). The slope was significantly different from zero ($F = 22.3$, $P < 0.001$). SGR did not depend on concentration or water flow rate alone. These results suggest that concentration and flow rate must be considered together (*i.e.*, as N-flux) in order to understand nutrient uptake and nitrogen-limited growth in *Gracilaria*.

The support of the National Wildlife Federation and Sigma Xi, The Scientific Research Society, as well as the able assistance of C. Errera and S. Nolan, is gratefully acknowledged.

## *The global circulation and distribution of DDT.* DORIA R. GORDON (The Ecosystems Center, Marine Biological Laboratory).

The release of persistent pollutants continues to increase despite evidence of negative effects in humans and other organisms. Use of the insecticide DDT is currently extensive in the tropics. This work has focussed on the consequences of a southward shift in application of this compound.

A review of investigations reveals that all organisms and segments of the biosphere studied, including air, water, and sediments, contain detectable levels of DDT residues. The troposphere has an average background concentration of about 0.1 parts per trillion. Nonetheless, longterm studies indicate that concentrations of DDT are decreasing in northern regions and are either stable or increasing in the tropics and Southern Hemisphere.

A simple model has been developed to examine the global circulation of DDT residues. The model reproduced the trends of contamination observed in nature. Residence times of DDT are years to decades. Such persistence ensures high levels of residues in the global system and the continuation of longterm dispersal. The analysis was limited because countries are not required to disclose the actual amounts of the insecticide that are produced or consumed. Thus, the quantity and distribution of the DDT released to the global environment is unknown.

Continued investigation of DDT's fluxes is appropriate to determine where accumulation and effects will occur. Critical gaps in knowledge result from the lack of sampling in the Southern Hemisphere and absence of information on production and use. Experience with the model suggests that fluxes in the tropics are more rapid than in the temperate zone, reducing the hazard involved. Longterm global monitoring of both specific species and abiotic reservoirs should provide indications of trends and increase understanding of the implications involved with the release of similar compounds, such as PCBs.

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## *Density effects on growth and survival of* Salicornia bigelovii *and* S. europaea. JEAN M. HARTMAN (Marine Biological Laboratory) AND MARLIES ENGLER.

Two annual glassworts, *Salicornia bigelovii* and *S. europaea* (Chenopodiaceae), are common in Great Sippewissett Salt Marsh, MA. These plants are found primarily in areas of reduced grass cover, where the density and size of the plants vary greatly. We tested the effect of density on the growth and survival in areas with dense, monotypic stands. For each species, we selected five 0.25 $m^2$ plots, over a 10 cm range of elevations, which were divided into 25 0.01 $m^2$ quadrats. The density within each quadrat was reduced to a level between 2 and 200 seedlings in early June. This design allowed us to test for between plot effects as well as density effects. In mid-July and mid-August, we counted the number of plants surviving in each quadrat and weighed subsamples of each density treatment.

Growth, measured as the average weight per individual, showed no significant density-dependent effect in either species. The dry weight of individual plants varied greatly, but no predictable pattern emerged among individuals from different density treatments. Survival rate of *S. bigelovii* showed a highly significant density-dependent effect, determined using a linear regression model. However, the data are scattered and only 25% of the variability can be explained by this factor. *S. europaea* showed no significant density-dependent growth characteristics. Analysis of each set of five experimental plots showed that elevation is significantly correlated with growth and survival. Also, we noted that intense herbivory was common in the *S. europaea* plots.

We conclude that the size and survival rate of these species is more strongly determined by site effects than by intraspecific interactions. Site effects include environmental characteristics such as elevation and density-independent biotic effects such as herbivory.

This work was supported by NSF grant OCE76-19278 to H. Caswell. Marge Taylor assisted in data collection.

### *Denitrifying bacteria in the Great Sippewissett Salt Marsh: their numbers, diversity, and distribution.* M. E. HEIMBROOK (Dept. Biological Sciences, Univ. Northern Colorado, Greeley, Colorado 80639) AND J. S. POINDEXTER.

Denitrification in the salt marsh represents a major nitrogen loss from this ecosystem (Valiela and Teal 1979, *Nature* **280**: 652). Sediments from the creek beds, pans, high and low marsh areas covered with *Spartina alterniflora*, and microbial mats sampled in the present studies yielded denitrifying organisms from cores taken as deep as 90 cm. Gram-negative, aerobic to microaerophilic, polarly flagellated coccobacilli, rods, vibrios, and spirilla were isolated as denitrifiers from enrichment cultures in LANA medium (1% $KNO_3$, 0.1% sodium lactate, 0.1% sodium acetate, 0.02% $NH_4Cl$, 0.05 m*M* $Na_2HPO_4$, 0.05 m*M* $KH_2PO_4$, in 80% sea water and adjusted to pH 8.0). Marsh sediments diluted ten-fold were incubated in semisolid LANA medium in serum-stoppered tubes with 0.1 atmosphere of acetylene in air. Acetylene blocks the reduction of $N_2O$ to $N_2$ (Yoshinari *et al.* 1977, *Soil Biol. Biochem.* **9**: 177) and caused the accumulation of $N_2O$, an intermediate in denitrification, to reach levels detectable by gas chromatography. Most probable number (MPN) estimates of denitrifiers in the semisolid LANA shake cultures ranged from 50/g wet sediment from the creek beds, to 1800/g in the sediments under the cyanobacterial mats. Populations of heterotrophic bacteria able to grow in the medium under these conditions ranged from 3400/g in the wet pan sediment to 120,000/g in the creek bed sediments. The denitrifiers in the sediments under the cyanobacterial mat represented 20% of the bacteria growing on LANA medium, but only 0.04% of the heterotrophs from the creek bed were denitrifiers. The numbers implied by the shake tube MPN procedure may be underestimated by two orders of magnitude because of heat sensitivity of the aquatic bacteria. However, the $N_2O$ detection method offers promise as a rapid and sensitive method for detecting denitrifiers. Additionally, the release of $CO_2$ by growing organisms serves as a positive internal standard for growth and for the gas chromatographic analysis of the metabolic products.

Parts of this research were supported by the Foundation for Microbiology and NASA NAGW-306.

### *Regeneration and maturation in two sympatric* Capitella *(Polychaeta) sibling species.* SUSAN D. HILL (Michigan State University), JUDITH P. GRASSLE, AND SUSAN W. MILLS.

The rapidity with which certain sibling species in the polychaete genus *Capitella* can reproduce and increase their population densities in disturbed marine habitats is well known. Sampling these populations has commonly shown that a high proportion of *Capitella* show evidence of tail regeneration. Laboratory experiments have been conducted to investigate the effects of regeneration on maturation and fecundity in the co-occurring *Capitella* species I and II. Regeneration was initiated by amputating tails in anesthetized worms of known age and parentage. Weekly observations were made on the reproductive state and number of regenerated tail segments in individual worms.

(1) In *Capitella* species I juveniles at ≈25°C tail regeneration and sexual maturation proceeded simultaneously. (2) At 20°C, *Capitella* species I males isolated to promote switching to a hermaphrodite mode went through oogenesis equally rapidly whether they were regenerating or not. (3) At ≈25°C, *Capitella* species I females allowed to reproduce 4–5 times regenerated tail segments at the same rate and to the same degree as females that were in the early stages of first oogenesis. That is, in *Capitella* species I the process of regeneration does not appreciably affect the processes of maturation and oogenesis.

(4) In *Capitella* species II at 20°C, regeneration significantly slowed the rate of sexual maturation from the late juvenile stage in developing males and females. (5) In a separate experiment regenerating *Capitella* species II females had a significantly lower fecundity over an 8-week period than nonregenerating worms. That is, in *Capitella* species II regeneration appears to occur at the expense of reproduction, delaying maturation and reducing fecundity.

In the field heavy predation produces a high proportion of regenerating worms. We hypothesize that the resultant delayed sexual maturation and reduced fecundity in *Capitella* species II, but not in *Capitella* species I, may account in part for the differential success of these two species in colonizing new habitats.

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### *Effect of nitrogen in litter and in ambient water on microbial respiration in* Spartina *decomposing in laboratory microcosms.* Andrew C. Marinucci, John E. Hobbie, Teresa L. Corliss, and John V. K. Helfrich (The Ecosystems Center, Marine Biological Laboratory).

The effects of nitrogen in ambient water and in litter on the decomposition of *Spartina alterniflora* were tested with laboratory percolators. The $CO_2$ respired by microbes in these systems was used as an indicator of decomposition. The systems were operated for 133 days at 22–24°C, contained 10 g of air-dried shredded litter, and were kept moist with artificial sea water (24‰) at 0.28–0.32 ml/mm. Outflow air was trapped in 0.2 *N* KOH and titrated periodically to determine $CO_2$ production. Details of operation have been previously described (Marinucci 1982, *Biol. Bull.* **163**: 53–69). The inflow water contained 0, 10, or 100 mg/l N as $NH_4Cl$. The litter was collected from areas at a salt marsh that had been fertilized (Valiella *et al.* 1975, *J. Appl. Ecol.* **12**: 973–982); as a result, the litter in the experiments contained 0.86, 0.95, 1.22 and 1.33 %N/g AFDW.

The results of this experiment in which effects of four levels of internal N were measured at three levels of N in the surrounding water demonstrated that increasing the litter N caused a direct increase in total $CO_2$ production. The differences among $CO_2$ produced from the various litter types resulted mainly from differences in rate of production in the first 60 days. The $CO_2$ production rate was the same for all litter types after day 60. Ambient water N had a smaller impact on total $CO_2$ production. Maximum total $CO_2$ production occurred in litter incubated with 10 mg N/l. However, this maximum production resulted from a very high rate in the first 35 days. After day 60, litter receiving 0 mg N/l water had the highest rate of $CO_2$ evolution with 10 mg N/l next lower and 100 mg N/l the lowest. Overall conclusions were that litter N rather than water N had the greater impact on decomposition.

This work was supported by NSF DEB 79-05127.

### *Potential nitrification rates in a salt marsh.* Erich R. Marzolf (Colorado College, Colorado Springs, CO 80903)

Nitrification is an important process in the cycling of nitrogen in coastal marine sediments. Potential nitrification rates were measured in sediments from Great Sippewissett Marsh, Falmouth, Massachusetts in August, 1982. Sediments were suspended in ammonia-enriched, aerated sea water and shaken. Temperature was maintained at 23–27°C. Nitrification was measured through the formation of nitrate and its subsequent reduction to nitrite. Rates were highest in the top 2cm and decreased exponentially with depth. Nitrification rates were calculated for sediments from various habitats within the marsh. Creek bank sediments had the highest rate (260 mg $N\text{-}NO_3 \cdot h^{-1} \cdot m^{-2}$), while sediments from sandy and muddy creek bottoms had rates of 16.6 and 9.0 mg $N\text{-}NO_3 \cdot h^{-1} \cdot m^{-2}$, respectively. A total nitrification rate of 26 kg $N\text{-}NO_3 \cdot h^{-1}$ was calculated for the marsh based on the surface area of each habitat. This total rate is sufficient to account for the estimated difference between nitrate import and combined nitrogen gas and nitrate export (4740 kg $N\text{-}NO_3$) in Great Sippewissett Marsh.

### *The effect of habitat structure on the predator-prey relationship between the green crab,* Carcinus maenas, *and the blue mussel,* Mytilus edulis. Eugene C. Revelas (Marine Sciences Research Center, SUNY—Stony Brook).

Blue mussels, *Mytilus edulis*, abound in Nauset Harbor, Orleans, MA (Cape Cod), both exposed on the mudflats and concealed within a bordering *Spartina alterniflora/Fucus vesiculosus* marsh. The green crab, *Carcinus maenas*, a voracious predator, is abundant on these mussel beds. The effect of the marsh habitat on this predator-prey relationship was investigated in seawater tables in the laboratory. *Carcinus* of various sizes were starved for 24 hours and then allowed to forage individually on equal numbers of different sized mussels in both aquaria devoid of vegetation (representing the mudflat habitat) and in a simulated "marsh." The "marsh" was constructed by sticking plastic straws (*Spartina*) into holes in a piece of plywood and securing *Fucus*, collected in the field, around the straws. During each foraging experiment mussels eaten were replaced to maintain constant prey density.

*Carcinus* (3–5 cm in carapace width) were found to predominate in the field based on three ½-hour searches. In the laboratory, *Carcinus* (3–5 cm) consumed mussels 0–3 cm in length. The predation rate (number of mussels eaten $\cdot$ crab$^{-1}$ $\cdot$ day$^{-1}$) in the "marsh" was 70% lower than in the "mudflat," apparently as a result of reduced predator-prey encounters. The size-frequency distributions of the marsh and mudflat *Mytilus* populations in Nauset Harbor (estimated by measuring all mussels within randomly tossed 10 × 10 cm quadrats) are explained by these laboratory results. Mussels 0–3 cm in length are significantly more abundant in the marsh (N = 132) than on the mudflat (N = 35). Also, both populations show enhanced numbers of individuals beginning at 3 cm. This *Mytilus* population has refuges from *Carcinus* predation both in space and in size.

The agreement between the laboratory results and the field observations suggests that these laboratory predator-prey manipulations reflect natural interactions. These data indicate that natural structural complexities drastically alter the predator-prey relationship between *Carcinus* and *Mytilus.*

### *The effects of sulfide on cyanobacterial photosynthesis in marine microbial mats.* THOMAS M. SCHMIDT, (Ohio State University) AND RICHARD W. CASTENHOLZ.

The cyanobacterial or blue-green algal mats of the Little Sippewissett Marsh in Falmouth, Massachusetts are dominated by two cyanobacteria, *Microcoleus chthonoplastes* and *Lyngbya aestuarii.* The concentration of hydrogen sulfide in these mats reaches at least 1 m*M* during the summer morning hours. This study examines the effects of sulfide on the photosynthetic apparatus of these cyanobacteria.

Cores of the mat were pre-incubated in the light for 3 hours in the presence or absence of 1 m*M* sodium sulfide, washed, and then assayed for their ability to photoassimilate $H^{14}CO_3$ in the presence and absence of sulfide. Field samples not pre-incubated with sulfide showed a 50% inhibition of $H^{14}CO_3$ fixation at 1 m*M* sulfide. This increased to 70% inhibition at 2 m*M* sulfide. $H^{14}CO_3$ fixation in field samples pre-incubated with sulfide was inhibited by 60% at 1 m*M* sulfide and 75% at 2 m*M* sulfide. The addition of DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) to field samples pre-incubated with or without sulfide, inhibited $H^{14}CO_3$ fixation by 90%. This inhibition was not decreased by the addition of 1 m*M* sulfide as would be expected if sulfide-dependent photosynthesis were taking place.

Pure cultures of *Lyngbya aestuarii* were pre-incubated in the same manner as the field samples. $H^{14}CO_3$ fixation in cultures previously unexposed to sulfide was inhibited by only 3% under 1 m*M* sulfide. When the culture was pre-incubated with sulfide, $H^{14}CO_3$ fixation was inhibited by 10% when again exposed to 1 m*M* sulfide. Two m*M* sulfide inhibited $H^{14}CO_3$ fixation by 50% regardless of previous exposure. DCMU again inhibited $H^{14}CO_3$ fixation by 90% in the presence or absence of sulfide.

Neither the field samples nor the *Lyngbya* culture showed any evidence of anoxygenic photosynthesis, and the field samples showed a low tolerance to sulfide. These results are somewhat unexpected since these mat organisms are subjected to frequent exposures to sulfide and might be expected to have retained or evolved a greater degree of sulfide tolerance or sulfide-dependent anoxygenic photosynthesis.

### *Wrack accumulation and vegetation structure in Great Sippewissett Salt Marsh.* EDWIN K. SILVERMAN AND JEAN M. HARTMAN (Marine Biological Laboratory).

A descriptive study was conducted in central Great Sippewissett Salt Marsh to compare the distributions and associated vegetation of wrack (dead plant material washed into the marsh by tides) and pannes (algae-covered or bare areas). This study is part of a larger project being conducted by one of us (J. Hartman), in which the main hypothesis is that wrack accumulation can cause panne formation.

Percent cover measurements inside a 0.04 m$^2$ quadrat were made every meter along twenty-two line transects. The transects were placed at ten meter intervals from the ocean to the marsh edge. Percent cover was sampled for 1781 quadrats. Tidal inundation was measured on 325 of the sample quadrats. Wrack covered 8.0% of the sample quadrats.

The relations of wrack and panne distribution were compared to tidal inundation and distance from the ocean. Most of the wrack, 85.9%, was found in a narrow band of tidal inundation levels between 13 and 33 cm. Most of the pannes, 74.3%, were found between 28 and 48 cm; 71.5% of the pannes were found outside the main band of wrack accumulation. A similar pattern was observed with distance from the ocean; 78.1% of pannes were located beyond the range of most of this year's wrack accumulation. Therefore, if wrack causes most pannes, its long-term distribution differs considerably from this year's accumulation.

The relation of wrack and pannes to vegetation structure was analyzed using R-type principal components analysis. Wrack quadrats did not correlate significantly with any principal component. A principal component consisting of increased short *Spartina alterniflora, Salicornia europea,* and *Salicornia biglovii,* and decreased large *S. alterniflora* correlated significantly with pannes for some of the sample transects, suggesting a generalized vegetation type for pannes.

This work was supported in part by NSF Grant OCE 76-19278 to Hal Caswell. E.K.S. was supported by a W.H.O.I. Summer Student Fellowship.

### *Role of shoot photosynthesis in root-rhizome respiration in* Zostera marina *L. (eelgrass).* ROBERT D. SMITH, WILLIAM C. DENNISON, AND RANDALL S. ALBERTE (The University of Chicago).

The majority of productive seagrasses grow in anoxic sediments in coastal waters. Because the nature of the respiratory behavior of the underground tissues in these species is essentially unknown, we examined the role of shoot photosynthesis in supporting aerobic respiration in the root-rhizome system of the temperate seagrass *Zostera marina* L. (eelgrass).

Uptake and release of oxygen from the root-rhizome system of *Zostera* was measured polargraphically in a two-chambered apparatus fitted with an oxygen electrode. Root-rhizome respiration rates averaged 15.8 (±0.3) nMol $O_2 \cdot h^{-1} \cdot mg^{-1}$ (dry wt). Upon illumination of the shoot, oxygen transport to the root-rhizome system began within 15 to 30 min and gave oxygen uptake and release rates of −6.2 to +7.4 nMol $O_2 \cdot h^{-1} \cdot mg^{-1}$ (root-rhizome dry wt) respectively, or −1.92 to +0.46 nMol $O_2 \cdot h^{-1} \cdot mg^{-1}$ (shoot dry wt). Within 10 to 20 min after the shoots were placed in the dark, oxygen transport to the root-rhizome ceased. Rates of oxygen transport to the root-rhizome during shoot photosynthesis ranged from 0.27 to 0.64 nMol $O_2 \cdot h^{-1} \cdot mg^{-1}$ (shoot dry wt). These results show that (1) shoot photosynthesis is responsible for oxygen transport to the root-rhizome system; (2) oxygen transport to the root-rhizome is rapidly initiated and terminated with changes in the shoot light regime; (3) shoot dry weight is highly correlated to the rate of oxygen transport to the root-rhizome; and (4) the shoot:root-rhizome ratio is highly correlated to the oxygen exchange rate with the sediments.

We have demonstrated that shoot biomass controls the supply of oxygen to the root-rhizome system of *Zostera*. This may explain the observed greater shoot biomass at depth under light-limited conditions. In addition, since daily light period and intensity for photosynthesis decreases with depth, the availability of oxygen for aerobic root-rhizome respiration also decreases along a depth gradient. Consequently, the period of root-rhizome anaerobiosis may influence the depth distribution of this species.

## *Semilunar spawning cycle in a Woods Hole population of* Fundulus heteroclitus. JEFFREY J. STODDARD (Dept. of Zoology, University of Wisconsin, Madison, WI 53703).

A spawning pattern synchronous with the semilunar tidal cycle has been demonstrated for a Massachusetts population of *Fundulus heteroclitus*, the common salt marsh killifish.

Samples were collected from 26 June to 9 August 1982 from the Great Sippewissett Marsh, Cape Cod. Measurements of gonadosomatic index [(wet gonad weight/body weight) × 100], percentage of oocytes in the final maturational stages, and mean number of mature oocytes per body weight showed clear increases immediately before each new and full moon. Collections of young-of-the-year fish showed discrete cohorts consistent with semilunar periodicity in spawning.

The spawning rhythm is of adaptive significance because it makes possible deposition of eggs within the high creekbank zone thus reducing their exposure to predators and strong currents.

## *Selection for moderately halophilic bacteria by gradual salinity increases.* A. VENTOSA (Dept. Microbiologia, Facultad de Farmacia, U. de Sevilla, Sevilla, Spain), J. S. POINDEXTER, AND W. S. REZNIKOFF.

Halophilic bacteria grow optimally in media containing 10% sea salts to saturation (>40%) and are usually isolated from hypersaline habitats. Because naturally occurring hypersaline sites are geographically discontinuous and generally result from concentration of sea water by solar evaporation, these studies addressed the question of whether oceanic water contained halophilic bacteria and so could serve as their medium of dispersal between hypersaline sites. One previous such study (Rodriguez-Valera *et al.* 1979, *Appl. Env. Microbiol.* **38:** 164) reported the isolation of halophilic bacteria from ocean waters off the coast of Spain within 15 km of onshore salterns. In this study, bacteria were collected by filtration from water of Vineyard Sound, whose shores lack extensive hypersaline sites. Two samples of 25 l each were filtered, and the bacteria from each sample were used to inoculate a medium containing a complex of organic nutrients. One sample was incubated initially with 3% sea salts and periodically received fresh medium containing salts adjusted to provide salts increments of 3%. The second sample was initiated at 10% sea salts, and salinity was increased in increments of 4%. Moderately halophilic bacteria, which accounted for fewer than $\frac{1}{500}$ of the viable bacteria present in the sample populations, were strongly favored in each culture when salinity reached 14–15%; they accounted for a majority of each population by the time the salts concentration reached 20%. Non-halophilic bacteria (both salt-tolerant and salt-dependent) decreased in numbers and diversity when salts concentrations reached 14–15%; at higher salinities, screening of clones of putative "marine bacteria" revealed them to be moderate halophiles able to grow over a wide (3–20%) salinity range. The quantitative changes in both cultures, which proceeded to higher salinities on different schedules, revealed that salinity alone accounted for enrichment of moderate halophiles. The results of these laboratory studies imply that halophilic bacteria of natural hypersaline sites are derived from sea water, and are enriched during the course of solar evaporation. Further, they predict that moderate halophiles become predominant in such sites by the time 80% of the water has evaporated and salts are at approximately one-third saturation. A collection of moderately halophilic bacteria has been accumulated for characterization.

Parts of this research were supported by the Foundation for Microbiology, and NASA NAGW-306.

### *The development and geomorphology of Great Sippewissett Marsh (Falmouth, MA): the Redfield model revisited.* MARC WEISSBURG, ALLYSON SENIE, GEORGE KOWALLIS, AND JOSEF TREGGOR (Dept. of Biology, Central Connecticut State College, New Britain, CT 06050)

The important ecological role of salt marshes has resulted in their being the focus of intensive scientific study, little of which has been concerned with marsh ontogeny. The notable exception is the elegant study of the Barnstable (MA) Marsh by Arthur Redfield (1965, *Science* **147**: 50–55). Utilizing data from sub-surface soundings and analysis of water content, organic content, and floral dominance in core samples we have developed a model of the Great Sippewissett Marsh.

Soundings at 670 points along three major transects revealed peat depths of 23–653 cm. Large scale depressions occurred along the upland margins while oscillations in topography and depth of peat decreased towards the sea. Coring sites were selected with reference to sub-surface topography and 26 cores were exuded. Based upon the percent of water and organic content (OC), sediment horizons were characterized as high marsh (60–90% $H_2O$; 35–69% OC), intertidal (30–60% $H_2O$; 2–25% OC), or fresh/brackish (85–92% $H_2O$; 70–90% OC). Visual inspection of the roots and rhizomes provided additional criteria for identification. Saltmarsh peat extended to depths of 400 cm and fresh/brackish peat to 650 cm.

Based on the depth of saltmarsh peat, the age was approximately 2900 years and generally followed the Redfield model of development. Certain differences were evident: Great Sippewissett developed through the establishment of saltmarsh islands which gradually coalesced into a contiguous high marsh plain, while Barnstable Marsh expanded from fringe areas. The colonization of upland areas occurred relatively later in the development of Great Sippewissett than of Barnstable.

Factors responsible for these differences were variation of surface topography and the development of fresh/brackish water wetlands over a substantial part of the abutting uplands prior to the commencement of saltmarsh development at Great Sippewissett Marsh.

It is clear that localized events play a major role in saltmarsh ontogeny and must be considered in any model of saltmarsh development.

### *Germination properties of a marine spore-forming bacterium.* P. WIER (Dept. EPO Biology, Univ. of Colorado, Boulder), A. KEYNAN, AND H. O. HALVORSON.

Pigmented aerobic spore-forming bacteria were isolated from the Sippewissett Marsh (Singer and Leadbetter 1974, *Biol. Bull.* **147**: 499) and from the marshes of the German North Sea (Fahmy 1978, Ph.D. Thesis, Göttingen). While there is evidence concerning the marine nature of these organisms, their germination properties are unknown. The germination properties of one such isolate (Hamlett, 1981) was investigated. This strain sporulates well on Zobells medium and produces a carotenoid pigment with maximum absorption at 492 nm. Spores were purified following lysis of the vegetative cells in distilled water and density centrifugation through 50 to 60% renografin. Optimal germination required a short heat activation (10 min at 60°C), pH 7.8, and 0.3 m$M$ adenosine, 85 m$M$ $Na^+$, and about 70 m$M$ $NH_4^+$. No germination occurs with either $Na^+$ or $NH_4^+$ alone. $NH_4^+$ could partly replace $Na^+$ but was much less effective ($K_m$ $NH_4^+$ = 214 m$M$; $K_m$ $Na^+$ = 14.7 m$M$). The divalent cation and dipicolinic acid (DPA) contents of 3A10 and two related marine spore-forming isolates were similar to those of terrestrial strains (DPA, 7.5–12.1%; $Ca^{++}$, 2.1–2.7%; $Mg^{++}$, 0.17–0.26%). The unique properties of these marine spore-formers are the stimulation of germination by $Na^+$, the requirement of $NH_4^+$ for germination, and the ability of sporulating cells to concentrate $Ca^{++}$ in a high $Mg^{++}$ environment.

### *A comparative study of anoxic decomposition in salt and freshwater marshes.* JOANNE WILLEY AND ROBERT W. HOWARTH (Ecosystems Center, Marine Biological Laboratory).

Anoxic decomposition represents 75–90% of total sediment metabolism in saltmarsh systems. The importance of anaerobic degradation in freshwater marshes is less well known. Anaerobic mineralization of organic matter is said to proceed at a uniformly rate than aerobic. However, anoxic decomposition can be more rapid than aerobic under some circumstances. Most energy from anaerobically decayed organic substrate is conserved as inorganic endproducts (sulfides or methane). The resulting low assimilatory efficiencies dictate that more organic substrate (relative to aerobic decomposition) is degraded to maintain a given microbial biomass. Thus, nutrient limitation of decomposition is less likely anaerobically than aerobically.

To evaluate belowground decomposition in salt and freshwater marshes, litter bags filled with one of four substrates were buried in May 1982 at three depths, 5, 15, and 25 cm, in the acidic sediment of a tidal freshwater marsh at North River and the reduced sediment at Great Sippewissett Salt Marsh.

Substrates used were *Typha* (dominant grass at North River) roots and rhizomes, *Typha* shoots and stems, and the same structures of *Spartina patens* (abundant at Sippewissett).

Replicate litter bags collected at 4 and 12 weeks demonstrate the importance of substrate quality and general lack of environmental mediation for the first 3 months of decomposition. Weight losses correlated with species and structure but not with burial depth or location. Decay rates ranged from −0.71 ± 0.62 for *Spartina* roots to −4.53 ± 0.95 for *Typha* roots. Nutrient dynamics reflected original litter content. The only samples to mineralize nitrogen, *Typha* shoots and stems, were also highest in original N concentration (C/N = 12.28 vs. about 15 for both roots and 25.56 for *Spartina* stems). Likewise, phosphorus-rich *Typha* components lost phosphorus, while phosphorus-poor *Spartina* immobilized P. Control of degradation as determined by marsh or depth of incubation may become more evident in the remaining nine months as litter composition becomes less variable.

### *Microbial colonization of filter paper incubated in saltmarsh sediments as observed by scanning electron microscopy.* N. WOGRIN (University of Massachusetts), J. S. POINDEXTER, AND E. P. GREENBERG.

Anaerobic cellulose decomposition in sediments of the Great Sippewissett Marsh and the School Street Marsh, Massachusetts, was studied using scanning electron microscopy (SEM) of filter paper incubated *in situ*. Pieces of filter paper were sandwiched between microscope slides and implanted in the top five centimeters of anaerobic marsh sediment. Filter paper samples were removed for observation after incubation periods of 3, 9, 21, and 29 days. Bacterial colonization of the filter paper incubated in the South Street Marsh sediment was apparent after nine days of incubation. Each microcolony consisted of cells of homogeneous morphology, and it is assumed that the organisms that persisted through preparation of the filter paper for SEM were those that were capable of stable attachment to the cellulose fibers. Morphotypes occurring in the microcolonies included ring-shaped cells (0.5 $\mu$m cell diameter), horseshoe and helical cells (0.5 $\mu$m cell diameter), and long rods (5–8 $\mu$m × 0.5 $\mu$m) that appeared to be flexible. Cells of each of these types had rounded poles, and in many microcolonies terminal swellings suggestive of endospores were observable. Given the anaerobic conditions of incubation, the presence of such terminal swellings suggests that these organisms are clostridia. Spirochete-like organisms and microcolonies of cocci were also occasionally observed. Cell types similar to those detected by SEM were observed when samples of the incubated filter paper were stained with acridine orange and viewed with epifluorescence microscopy. In addition to the morphologies described above, this technique revealed the presence of motile cells: vibrios, spirilla, and spirochete-like organisms. These were not attached to the filter paper and may have been lost during preparation for SEM. By the combined application of two types of microscopy, two populations were found associated with the cellulose during its deterioration under anaerobic conditions: microcolonies that appeared to have a stable attachment to the cellulose fibers, and non-adhering, motile associates.

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## FERTILIZATION AND DEVELOPMENT

### *Stimulus/response coupling in sponge aggregation: evidence for calcium as an intracellular messenger.* CATHLEEN ANDERSON, ABBY M. RICH, ADAM DICKER, PHILIP DUNHAM, AND GERALD WEISSMANN (Marine Biological Laboratory).

Aggregation of dissociated sponge cells (*Microciona prolifera*) has been proposed as a model for cell-cell recognition mediated by a specific proteoglycan aggregation factor (MAF). To test whether sponge cells undergo stimulus/response coupling in which intracellular $Ca^{++}$ is a messenger, mechanically dissociated cells were studied in a Payton aggregometer conventionally employed for kinetic analysis of aggregation of platelets and neutrophils. Changes in light transmission paralleled aggregation as judged by light, scanning, and transmission electron microscopy (EM). Cells ($2 \times 10^8$/ml) were equilibrated (30–60 min) in $Ca^{++}$-, $Mg^{++}$-free sea water (pH 7.8) with EDTA to deplete cells of $Ca^{++}$ and to inactivate soluble MAF. In the presence, but not absence, of $Ca^{++}$ (>5 m*M*) partially purified MAF (from Dr. M. Burger) aggregated both living and glutaraldehyde-fixed cells. MAF remained associated with the surface of EDTA-treated cells judged by their aggregation in response to anti-MAF, but not pre-immune serum. Evidence for a messenger role of intracellular $Ca^{++}$ was the following: 1) Addition of $Ca^{++}$ (>2.5 m*M*) to $Ca^{++}$-depleted cells induced aggregation that varied directly with the $Ca^{++}$ concentration. 2) Addition of calcium ionophores (A23187, ionomycin; >5 $\mu$*M*) caused aggregation which varied with extracellular $Ca^{++}$ and far exceeded that provoked by $Ca^{++}$ alone. Glutaraldehyde-fixed cells did not respond to ionophores ± Ca. 3) Calcium antagonists inhibited aggregation. These included a napthalene sulphonamide inhibitor of the Ca-calmodulin complex (W-7; >15 $\mu M$), a calcium channel blocker (verapamil; >100 $\mu M$) and three non-steroidal anti-inflammatory agents (indomethacin, ibuprofen, piroxicam; >50 $\mu M$). Cells remained viable in all circumstances (Trypan Blue exclusion; supravital staining; transmission EM). Results indicate that early events (0–5 min) of sponge aggregation can be quantified by a continuous recording technique, and that it is not simply the passive response of an inert cell to an extracellular proteoglycan. Rather, the sponge, like the platelet or neutrophil, recognizes surface ligands to which it responds by calcium-dependent stimulus-response coupling.

### *The effects of quercetin and ionophore A23187 on meiosis initiation in* Spisula *and* Asterias *oocytes.* William R. Eckberg (Department of Zoology, Howard University, Washington, D. C. 20059).

To examine the roles of calcium and calcium sequestration in meiosis initiation, we treated oocytes of *Spisula* and *Asterias* with quercetin, an ATPase inhibitor. Isotonic $CaCl_2$ initiated GVBD in *Spisula* oocytes only in the presence of quercetin. This suggests that quercetin initiates GVBD by inhibition of calcium sequestration. However, when the oocytes were treated in sea water, meiosis was not initiated, even when excess KCl was added in amounts below the threshold for parthenogenesis. High doses of quercetin blocked GVBD but not fertilization envelope elevation when they were induced by ionophore or excess KCl. Therefore the drug can have an additional inhibitory effect on GVBD. Kinetic studies showed that quercetin inhibited a relatively early event in GVBD induction. Quercetin also inhibited fertilization.

When starfish oocytes were treated with relatively high concentrations of quercetin, 1-methyl adenine (1-MA) stimulation of GVBD was blocked. When 1-MA was absent or present in subthreshold concentrations, lower concentrations of quercetin stimulated meiosis somewhat.

Treatment of *Spisula* oocytes with ionophore resulted in egg activation as demonstrated by fertilization envelope elevation, GVBD, and polar body formation. This result was dependent upon extracellular calcium. This result, together with the quercetin results, suggests that intracellular calcium in *Spisula* oocytes is exchangeable. Ionophore activated *Asterias* oocytes as shown by fertilization envelope elevation, but failed to initiate GVBD.

These results further indicate the importance of calcium sequestration in the maintenance of the germinal vesicle, but they also show that quercetin can have other effects on GVBD than the stimulatory effect previously shown.

### *On the role of maternal mRNA in sea urchins: studies of a protein which appears to be destroyed at a particular point during each cell division cycle.* Tom Evans, Tim Hunt, and Jim Youngblom (Physiology Course, MBL).

We have reinvestigated the pattern of protein synthesis after activation of eggs of the sea urchin *Arbacia punctulata* with sperm, $NH_4Cl$, or A23187. Eggs were labeled continuously with $^{35}S$-methionine, samples taken every 10 minutes, and the pattern of protein synthesis analyzed on SDS-polyacrylamide gels. Autoradiography of these gels revealed a heavily labeled protein, $M_r$ 55,000, which showed striking behavior as development proceeded: each time the eggs divided, it disappeared. Its synthesis is barely detectable in unfertilized eggs, although they contain high levels of mRNA for this protein, which we call "cyclin." Cyclin has the following additional properties: (1) It disappears completely after inhibition of protein synthesis by emetine. (2) It is synthesized at a constant rate as measured by successive 10-minute pulses with $^{35}S$-methionine during the first 100 minutes of development. (3) Cyclin does not bind to a monoclonal anti-tubulin antibody which reacts strongly with *Arbacia* tubulin. (4) In the presence of inhibitors of cell division, $10^{-4}$ $M$ colchicine, $10^{-5}$ $M$ taxol, or $4 \times 10^{-6}$ $M$ cytochalasin D the level of cyclin rose normally, but disappeared very slowly. (5) Activation of protein synthesis with 10 m$M$ $NH_4Cl$ led to the continuous accumulation of cyclin with no sign of breakdown over a period of 2 hours. In contrast, A23187 gave a pattern of cyclin synthesis and breakdown very similar to that produced in the presence of the inhibitors mentioned above in (3).

Preliminary experiments show that proteins which exhibit similar properties are found in the urchin *Lytechinus pictus* and the clam *Spisula solidissima.*

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### *The ontogeny of the fertilization site in* Hydractinia echinata (hydrozoa). Gary Freeman (University of Texas at Austin).

Sperm will only fuse with hydrozoan eggs at the site of polar body formation (Freeman and Miller 1982, *Develop. Biol.* in press). This suggests that there is a special cell surface and/or cell membrane differentiation at this site.

The time during oocyte maturation when this site forms was established by adding sperm to oocytes which are at different stages of the process of maturation. After a 5 minute exposure to sperm, those sperm that had not been incorporated into the oocyte were destroyed with a 0.0005% SDS solution. The process of maturation was allowed to go to completion and sperm-egg fusion was assayed by establishing whether or not these eggs cleaved. Only eggs treated with sperm after second polar body formation cleaved.

The role of the oocyte nucleus in setting up the fertilization site was examined by moving the germinal vesicle or meiotic apparatus to a new position at different stages of oocyte maturation and establishing whether the displacement of the nucleus also displaced the site of fertilization. This was done by centrifuging the oocytes at 3000 × *g*. Prior to centrifugation the initial position of the oocyte nucleus was marked with the vital dye nile blue A. Centrifugation stratifies the contents of the oocyte into a centripetal pigment zone, a clear zone, and a centrifugal yolk-filled zone. The oocyte nucleus is always found in the clear zone just below the pigment layer. Those oocytes were selected where the mark and the pigment cap were not congruent. After the process of maturation was completed the oocyte was cut into fragments in such a way that one fragment contained the stain mark and the other fragment contained the pigment zone and most of the clear cytoplasm. Sperm was added to each fragment and fertilization was assayed by monitoring cleavage. One can move the site of fertilization by changing the position of the nucleus at any time prior to second polar body formation. After this time period the site of fertilization is fixed.

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## *An unexpectedly steep developmental gradient in* Asterias forbesi *embryos induced by anoxia.* S. INOUÉ, S. B. POTREBIC, C. R. BROWN, AND D. A. LUTZ (Marine Biological Laboratory).

Fertilized eggs from the starfish *Asterias forbesi*, sandwiched between slide and coverslip, crowded into a monolayer in filtered sea water and surrounded by a sealed air space, generate a steep developmental gradient. Observed *ca.* 15 hours after fertilization, a culture drop with diameter greater than 5 mm and density greater than 19 embryos per mm$^2$ has fertilized eggs in the center which have not cleaved. From the center outward are concentric rings of cells which have divided 1, 2, 3 . . . 9 times. Even in a single approx. 150 $\mu$m diameter embryo, cells on the inside may be arrested two division cycles earlier than the outer cells. The wave of division arrest is propagated outward radially at a rate of approximately 60 $\mu$m per hour. Finally one reaches an outside rim of 1.5–2.2 mm in which all embryos have hatched as swimming blastulas simultaneously with controls.

Anoxia was determined to cause the steep developmental gradient by the following experiments. 1) In a crowded hanging drop preparation no gradient developed. 2) Removing $CO_2$ with KOH did not reduce the gradient. 3) If $O_2$ is continuously perfused across the microdrop preparation, no gradient develops. 4) When a preparation is made containing 0.1% methylene blue, the dye is quickly reduced to its colorless form except in a 1 mm ring along the outer edge.

Even after long periods of anoxic arrest, the embryos in the center of the gradient do not lyse and can be revived by introduction of $O_2$. They then undergo successive divisions and develop. The appearance of such a sharp gradient of anoxic arrest and its reversibility suggest that self-generated redox gradients may well affect differentiation and development in embryos and tissues.

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## *Colcemid but not cytochalasin inhibits asymmetric nuclear positioning prior to unequal cell division.* DOUGLAS A. LUTZ AND SHINYA INOUÉ (Marine Biological Laboratory).

We investigated the mechanism of nuclear migration and spindle orientation that precedes unequal cleavage by interfering with microtubule and actin-microfilament assembly. In echinoid embryos, the nucleus and spindle are positioned asymmetrically and oriented appropriately prior to the unequal macromere/micromere forming division. Upon completion of the 3rd division, the vegetal blastomere nuclei of *Lytechinus variegatus* migrate from a central position to the vegetal pole (VP) cortex at a rate of 1.5–2.0 $\mu$m/min; a similar migration was also observed in vegetal blastomeres of *Clypeaster* and *Hemicentrotus* by Dan (1979, *Dev. Growth and Diff.* **21**: 527–535). In *Lytechinus*, the nucleus travels approximately 20 $\mu$m in 12 min at 21°C to a position 5–8 $\mu$m from the VP cortex. There the nuclear envelope breaks down and the mitotic spindle forms, already properly oriented. In addition to positional asymmetry, morphological asymmetry is present within the mitotic spindle; the peripheral aster is truncate whereas the internal aster is radiate. When Colcemid, which disassembles mitotic microtubules *in vivo*, is applied at the completion of the 3rd cleavage in concentrations greater than 1 $\mu M$, the vegetal blastomere nuclei do not migrate but remain centrally situated. If, at nuclear envelope breakdown, Colcemid is washed out and inactivated within the embryo by a short exposure to 366 nm illumination (Aronson and Inoué 1970, *J. Cell Biol.* **45**: 470–477), the spindle forms centrally and with symmetric asters. Anaphase ensues before appreciable spindle migration, so the division tends to produce daughter blastomers of nearly equal size. Concentrations of cytochalasins D(20 $\mu M$), E(1 $\mu M$) and dihydroxyB (1 $\mu M$), which interfere with actin assembly and relax cleavage furrows within 3 min, had no effect on nuclear migration; the rate of nuclear migration, final distance from VP cortex, and astral asymmetry are similar to controls. These data suggest that a microtuble-based or -mediated, but not an actin microfilament-based or -mediated motile system is responsible for the nuclear migration to its asymmetric position.

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### *The role of the germinal vesicle in the 1-methyladenine-induced changes in protein synthesis in* Asterias *oocytes.* Mark Q. Martindale and Bruce P. Brandhorst (McGill University).

*Asterias* oocytes undergo dramatic translationally mediated qualitative and quantitative changes in protein synthesis after induction of meiotic maturation by 1-methyladenine (1MA) (Rosenthal *et al.* 1982, *Develop. Biol.* **91**: 215–220). The beginning of these changes coincides with the breakdown of the germinal vesicle (GV). To investigate the role of the release of GV contents in these changes in protein synthesis we isolated nucleated and enucleated fragments of *Asterias* oocytes. Oocytes were collected in $Ca^{++}$-free sea water (CFSW pH 5.0) and filtered through cheesecloth to remove follicle cells and jelly coats. The oocytes were then layered on a discontinuous sucrose gradient consisting of an upper 1.5:1 (1 $M$ sucrose: CFSW) layer, a middle 4:1 layer, and a 1 $M$ sucrose cushion. The samples were centrifuged at 5000 rpm for 20 min at 8–10°C in a Beckman JA-13 swinging bucket rotor. The speed was then increased to 12,000 rpm for the last 20 min. Enucleated fragments were collected off of the 1 $M$ cushion and their purity established by phase contrast microscopy. Nucleated fragments were taken from the top of the 4:1 layer. All fragments were washed with filtered sea water and aliquots activated by addition of $3.0 \times 10^{-5}$ $M$ 1MA. Fragments and intact oocytes were labeled with $^{35}S$-methionine (0.5 mCi/ml; 1200 Ci/mMol) for 30 min and newly synthesized proteins compared by electrophoresis on 10% polyacrylamide gels containing SDS. Autoradiographs show that the proteins synthesized by both unactivated nucleated and enucleated fragments were indistinguishable from those of intact oocytes. Following addition of 1MA essentially identical changes in protein synthesis were observed for nucleated and enucleated fragments as well as activated intact oocytes. We conclude that maternal RNAs or translational factors required for the changes in protein synthesis are not sequestered in the GV. Thus, most, or all, of the maternal mRNAs becoming available for translation during maturation are stored in the cytoplasm of the oocyte.

This research was carried out in the Embryology Course which is supported by a grant from the N.I.H.

### *Effect of gossypol on* Arbacia *sperm ATPase.* Hideo Mohri (Department of Biology, University of Tokyo, Japan), Kyoko Matsuda, S. S. Koide, and Sheldon J. Segal.

Motility of *Arbacia* spermatozoa is inhibited by gossypol, and both pyruvate dehydrogenase and $Mg^{2+}$-ATPase activities of the sperm mitochondria are much reduced by this substance (Adeyemo *et al.* 1981, *Biol. Bull.* **161**: 333), suggesting that gossypol limits ATP supply to the motility apparatus of the spermatozoa. The present study determined whether or not gossypol directly affects the motility system of sea urchin spermatozoa.

Intact *Arbacia* spermatozoa immediately stop their movement when exposed to 0.3 m$M$ gossypol. At lower concentrations the effect is less pronounced. The ATP-induced motility of sperm demembranated with Triton X-100 is relatively insensitive to the action of gossypol. To achieve complete arrest of motility concentrations of gossypol as high as 1 m$M$ are required. The ATP-induced motility of demembranated sperm exposed to 0.3 m$M$ gossypol is as vigorous as that displayed by controls. When the spermatozoa are preincubated in 0.3 m$M$ gossypol for 10 min, and subjected subsequently to the demembranation and reactivation procedure, the demembranated sperm become vigorously motile. This result supports the postulate that gossypol limits the ATP supply to the sperm's motility apparatus.

To determine whether gossypol directly influences dynein ATPase activity, *Arbacia* spermatozoa were fractionated into the head-plus-midpiece and tail fractions. The tails were further demembranated to obtain the axonemes. $Mg^{2+}$-ATPase activities of all these fractions are inhibited by gossypol. At a concentration of about 50 $\mu M$, gossypol inhibits the enzymatic activities of these preparations by 50%. Finally, 21S dynein was extracted from *Arbacia* sperm axonemes and the effect of gossypol on its $Mg^{2+}$-ATPase activity was tested. The purified 21S dynein ATPase is inhibited by gossypol at a concentration of 2 $\mu M$. Thus, although gossypol would primarily affect the ATP-generating system *in vivo*, it also inhibits dynein ATPase.

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### *Effect of heat shock on nuclear RNP structure in mammalian cells.* CHRISTINE MAUTE MORGANELLI (Dartmouth College).

Heterogeneous nuclear RNA is normally complexed with a specific set of proteins, forming ribonucleoprotein particles termed hnRNP. These particles are likely to be involved in mRNA processing. Recently, it has been shown in cultured *Drosophila* cells that the assembly of hnRNA into hnRNP particles is blocked by heat shock (S. Mayrand and T. Pederson, personal communication). Because mammalian cells also show a heat shock effect on protein synthesis, it was of interest to determine whether hnRNP assembly is also altered in these cells by elevated temperature. HeLa or mouse erythroleukemia cells were pulse-labeled with $^3$H-uridine at 37°C or the desired elevated temperature, and hnRNP particles were isolated from nuclei by standard procedures. The protein content of these particles was analyzed by equilbrium centrifugation in $Cs_2SO_4$ density gradients. Heat shock altered the assembly of hnRNP in both HeLA and mouse erythroleukemia cells. HnRNP from control cells (37°C) banded at a density of 1.35 g/cm$^3$ (approx. 80% protein: 20% RNA), whereas after heat shock (39°–43°C) an increasing proportion of the particles banded at higher densities (1.45–1.60 g/cm$^3$), indicative of a greatly reduced protein content. Further results indicate that the shift in hnRNP structure is gradual at progressively higher temperatures, rather than an all-or-none response. The effect of heat shock on hnRNP is first observed at 39°C, whereas no inhibition of total hnRNA transcription occurs until 42°–43°C. The possibility arises that the blocked hnRNP assembly is related to altered post-transcriptional mRNA processing after heat shock. In particular, this condition might favor the processing of mRNA's that do not undergo splicing. It is noteworthy that most of the heat shock mRNA's lack intervening sequences.

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### *Synthesis of 5S RNA and tRNA in cleaving sea urchin embryos: effect of altering cell interactions.* ANNE F. O'MELIA (Department of Biology, George Mason University, Fairfax, VA 22030).

The synthesis of 5S RNA and of transfer RNA (tRNA) has been shown to occur as early as the 16- to 32-cell stage in cleaving sea urchin embryos (O'Melia 1979, *Develop. Growth and Differ.* **21:** 99–108). Rates of accumulation of newly made 5S RNA and tRNA per cell are highest during cleavage and decline about threefold during development to the pluteus stage (O'Melia 1979, *Differentiation* **15:** 97–105). The present study determined whether normal cell associations and interactions are necessary for 5S RNA and tRNA synthesis in cleaving embryos of the sea urchin, *Arbacia punctulata*. Cell interactions were altered: (1) by culturing cleaving embryos in evans blue, which induces animalization (ectodermalization), and in LiCl, which induces vegetalization (endo-mesodermalization) of whole sea urchin embryos; and (2) by culturing cells dissociated from cleaving embryos under conditions which prevent reaggregation. Control and experimental embryos and dissociated cells each were labeled from 3 h to 6 h post fertilization with guanosine-[8-$^3$H] and with L-[$^3$H-methyl]-methionine. Total cellular RNA was extracted using the cold (4°C)-phenol-sodium dodecyl sulfate method, and purified (LiCl-soluble) RNA preparations were fractionated by electrophoresis on 10% polyacrylamide gels. Rates of accumulation of newly made 5S RNA and of tRNA in control and in experimental embryos were calculated from the radioactivity coincident with the 5S RNA and with the tRNA absorbance peaks ($A_{260}$ nm) on each gel, from the known GMP composition of sea urchin 5S RNA and tRNA, and from the average specific radioactivity of the GTP precursor pool during the 3-h labeling period. The results show that rates of synthesis of 5S RNA and tRNA per embryo and per cell are similar in control embryos and in cleaving embryos cultured in the presence of animalizing and vegetalizing agents. In addition, cells dissociated from cleavage embryos retained the ability to synthesize 5S RNA and tRNA. These results suggest that normal cell associations and interactions are not necessary for the synthesis of 5S RNA and tRNA in cleaving sea urchin embryos.

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### *Vitellogenesis in the hepatopancreas and ovaries of* Carcinus maenas. JEANNE E. PAULUS AND HANS LAUFER (The Biological Sciences Group, The University of Connecticut, Storrs, CT).

The site(s) of synthesis of yolk proteins or their precursors has never been clarified in crustacea. It has been shown repeatedly that removing eyestalks of various crustaceans during their reproductive season stimulates ovarian growth and presumably vitellogenesis. Eyestalk ablation of mature female *C. maenas* during May, June, and early July increased the mean gonadal index from 1.1 to 4.0%, and the percentage of spawning females rose to 50% relative to 30% in unoperated controls. In controls 75% were non-vitellogenic, while 90% of the experimental group were vitellogenic 36 days after eyestalk removal.

Since potential sites of action of the ovary stimulating factor are the ovary and hepatopancreas, we have developed an *in vitro* system for assaying lipovitellin synthesis in these possible target tissues. Fragments of tissue are cultured in media consisting of 1.5 m*M* D-glucose, salts, antibiotics, and $^3$H-L-amino acids at 17°C up to 16 hours. The percentage lipovitellin synthesized, relative to total TCA-precipitable counts, is assayed by a double immunoprecipitation technique using lipovitellin-specific antibody produced in rabbits, and protein A of *Staphylococcus aureus* (Cowen I strain).

There is a developmental pattern of lipovitellin synthesis in the hepatopancreas and ovary. The hepatopancreas is most active in lipovitellin production during stage 3, when oocytes measure 0.2–0.4 mm in diameter. The synthesis of lipovitellin is greatest in the ovary at stage 4, characterized by oocytes 0.4–0.7 mm in diameter. The activity of the hepatopancreas is relatively low at this time.

This is the first report demonstrating conclusively the synthesis of lipovitellin, or its precursors, in the hepatopancreas. Furthermore, this synthesis coincides with the time when the ovary incorporates serum vitellogenins into developing oocytes. Before and after this stage of specific uptake, the ovary is the major contributor of lipovitellins to the oocyte.

This research was supported in part by grants from the National Science Foundation, the Institute of Water Resources, and The University of Connecticut Research Foundation.

### *A new method for preparing marine eggs for microinjection: the "fly paper" technique.* MARK BENNETT POCHAPIN, JEAN M. SANGER, AND JOSEPH W. SANGER (Department of Anatomy G/3, University of Pennsylvania School of Medicine, Philadelphia, PA 19104).

A simple method for microinjecting large numbers of sea urchin eggs was devised by attaching eggs to a poly-L-lysine-coated coverslip and placing it in an open Petri dish containing sea water. The eggs can be fertilized either before or after they are attached to the coverslip and will divide normally, hatch, and form plutei. In this way, specially constructed chambers are not required and the eggs can be microinjected from the top in the same manner as tissue culture cells. The seawater medium can be removed easily and replaced with artificial medium if desired, or the coverslip can be removed from the dish and mounted in order to flatten the cells for better visualization of the spindle. Preparation of coverslips requires that they be thoroughly cleaned in detergent and distilled water, then in 95% ethanol followed by vigorous rubbing with cheesecloth until the coverslip surface feels smooth. Approximately 20 drops of a freshly prepared poly-L-lysine (Sigma Chemical Co., mol wt > 300,000) solution (1 mg/ml) are added to the cleaned coverslips and allowed to stand for one hour. The coverslips are drained by touching one edge to a piece of filter paper and then air-dried and placed in 35-mm plastic Petri dishes. Eggs of *Arbacia punctulata*, shed into filtered sea water, were transfered with a Pasteur pipette to form a large drop on the coated coverslip. Within five minutes, the eggs settled from the drop and adhered to the coverslip, after which time more sea water was added to half-fill the Petri dish. The attached eggs could be fertilized in the dish by adding a drop of sperm suspended in sea water. Fertilization membranes formed normally and the excess sperm quickly stuck to the poly-L-lysine surface giving it the appearance of fly paper. Microinjection was accomplished by placing the Petri dish on the stage of an inverted microscope and injecting desired solutions into embryos or unfertilized eggs with the aid of a pressure regulator connected to a nitrogen tank. When Lucifer yellow was injected into unfertilized eggs that were subsequently fertilized, development proceeded normally to the pluteus stage where all cells contained Lucifer yellow. We believe that this method of microinjecting sea urchin eggs offers a relatively simple way of introducing a variety of agents into a large number of eggs or embryos in a short period of time, enabling the effects of the agents to be monitored during development.

This work was supported by funds from the National Institutes of Health. We are grateful to the Basic Research Support Grant Committees of the University of Pennsylvania for funds for some of the video cameras and recorders used in this work.

### *Sperm agglutinating factor isolated from* Spisula *oocytes.* EIMEI SATO, S. J. SEGAL, AND S. S. KOIDE (Population Council).

A membrane component present on the surface of *Spisula* oocytes was found to induce sperm agglutination. Purification and characterization of the oocyte surface component (OSC) were carried out. Several extraction media were tested at varying incubation times. The following media were used: (A) 1 *M* urea, 5 m*M* EDTA, 10 m*M* Tris · HCl, pH 7.4; (B) 1 *M* urea, 10 m*M* Tris · HCl, pH 7.4; (C) 5 m*M*

EDTA in artificial sea water (ASW). Oocytes incubated up to 15 min in media A or B at 22°C exclude the dye trypan blue indicating that they are viable. After 15 min, however, progressive staining of the oocytes occurs. Oocytes incubated in medium C for two hours or longer remain viable. Thus, exposure of oocytes to media containing 1 *M* urea for longer than 15 min results in disruption of the cell membrane.

Oocytes incubated in medium A or B, then washed with 400 m*M* Tris · HCl, pH 7.4, 2 m*M* $CaCl_2$ do not undergo germinal vesicle breakdown (GVBD) after exposure to sperm. However, GVBD is induced in these oocytes by exposure to 70 m*M* KCl. This suggests that urea treatment results in the removal or alteration of a membrane component involved in sperm-oocyte interaction.

Sperm added directly to medium A, B or C do not agglutinate. After oocytes are incubated in medium A or B, the ambient medium induces sperm agglutination; the clumps remain intact for at least one hour while the aggregate sperm retain motility. This observation indicates that a factor is extracted from oocytes which induces sperm agglutination. The *Spisula* oocyte extract does not agglutinate sperm of *Arbacia, Asterias, Ovalipes,* or *Chaetopterus.* The agglutinating factor is stable at 100°C for 15 min and is not denatured by freeze-drying. It forms a precipitate when dialyzed against distilled water and is destroyed by trypsin. It is precipitated by acetone and is not absorbed on charcoal. These characteristics suggest that the factor is a protein or a glycoprotein. Its MW is estimated to be about 15 to 25 K daltons on the basis of gel filtration on Sephadex G-100 and by dialysis procedures using cellulose tubings with defined MW cutoffs.

When the factor is purified by ammonium sulfate fractionation (30% saturation) followed by gel filtration on Sephadex G-100, four major peaks are obtained. The fractions comprising the second and third peaks possess sperm agglutinating activity at a concentration of 2.5 μg/ml.

Supported by the Rockefeller Foundation and NIH. E.S. is a postdoctoral fellow of the Rockefeller Foundation.

## *Indomethacin, an anti-inflammatory drug, promotes polyspermy in sea urchins.* H. SCHUEL, E. TRAEGER, R. SCHUEL, J. BOLDT, AND M. ALLIEGRO (SUNY—Buffalo, Buffalo, NY).

Sea urchin eggs release $H_2O_2$ during the cortical reaction at fertilization to help prevent polypspermy by inactivating excess sperm near the egg (Coburn *et al.* 1981, *Dev. Biol.* **84**: 235–238; Boldt *et al.* 1981, *Gamete Res.* **4**: 365–377). This process resembles the peroxidatic killing of bacteria by phagocytic leukocytes during inflammation. Associated with these reactions in leukocytes, arachidonic acid is oxidized via the cyclooxygenase pathway to produce prostaglandins and thromboxanes as well as oxygen-free radicals and $H_2O_2$. Indomethacin is a potent inhibitor of cyclooxygenase in leukocytes. Polyspermy results when *Arbacia punctualata* and *Strongylocentrotus purpuratus* eggs are fertilized in 10–100 μ*M* indomethacin. The incidence of polyspermy depends upon the concentration of indomethacin and the number of sperm in the cultures. Indomethacin must be present prior to completion of the cortical reaction to promote polyspermy. Sperm fertility is known to be reduced by $H_2O_2$. Indomethacin does not protect sperm from inactivation by $H_2O_2$, and does not inhibit the sperm peroxidase that uses egg-derived $H_2O_2$ to inactivate sperm. Indomethacin apparently acts directly on the eggs to promote polyspermy. Aspirin, which is a less potent cyclooxygenase inhibitor, does not promote polyspermy at 5 m*M* in 20 m*M* Tris-buffered sea water at pH 8.0. These results suggest that sea urchin eggs may oxidize arachidonic acid by cyclooxygenase to help assure monospermic fertilization.

Supported by NSF grant #PCM-82-01561.

## *A study of the heat shock response in early embryos of* Spisula solidissima. LAURIE E. STEPHENS (Physiology Course, MBL).

Many cell types and organisms exhibit a heat shock response in which normal cellular protein synthesis is reduced while the synthesis of a new set of proteins, termed the heat shock proteins, is induced. I have performed a preliminary characterization of the heat shock response in embryos of *Spisula solidissima.* This was done by following *in vivo* protein synthesis with $^{35}S$-methionine at both normal (21°C) and elevated temperatures. Samples were run on 15% acrylamide gels and autoradiographed. The heat shock response is most clearly observed after a one hour exposure to 31°C, although the response can also be elicited at temperatures ranging from 29° to 35°C. Four polypeptides of molecular weight 115,000, 72,000, 70,000, and 37,000 daltons appear within 15 minutes after raising the temperature. Following a reduction in temperature to 21°C, these proteins continue to be synthesized for up to four hours, although normal protein synthesis resumes within an hour under these conditions. I could detect no heat shock response in either oocytes or embryos prior to two hours after fertilization.

Heat treatment appears to initiate *de novo* synthesis of the mRNA for the heat shock proteins. Total RNA was extracted from embryos and assayed in the reticulocyte lysate translational system. No heat shock mRNA was detectable in control embryos, whereas the mRNA from heat-shocked embryos gave rise to heat-shocked proteins as the major translation products. Interestingly, however, synthesis of most if not all of the normal proteins was also specified by these preparations, although their synthesis was barely detectable in the heat-shocked embryos, suggesting the existence of regulation at the translational as well as the transcriptional level.

This work was supported by NIH training grant GM-31136-04 to the Physiology Course. Thanks also to Tim Hunt, Eric Rosenthal, and Andrew Murray for invaluable guidance and advice.

## *A low molecular weight subunit of the aggregation factor complex of* Microciona prolifera *that stoichiometrically binds to and inhibits the intact aggregation factor.* Pachara Verakalasa and Tom Humphreys (University of Hawaii).

When $Ca^{++}$ is removed, the 20 million dalton aggregation factor (AF) complex of *M. prolifera* dissociates into subunits which inhibit aggregation. Inhibition was measured by defining one unit as the amount required to suppress aggregation with 4 units of AF for 30 min at 22°C. Four units of AF when dissociated gave one unit of inhibition. Ethanol precipitation and permeation chromatography revealed ethanol-soluble inhibitory subunits (IS) of less than 10,000 daltons which contain less than 5% of the protein and polysaccharide of the original complex. We postulated that the IS is a monovalent binding site of the complex which binds to intact AF and prevents the AF-AF interaction necessary for cell aggregation. Such binding was shown by mixing AF and IS and passing the mixture over a 3000 Å micropore glass bead column at 22° in $10^{-2}$ or $10^{-3}$ *M* $Ca^{++}$. On this column AF is excluded and is separated from IS which is fully included. When 64 units of AF is mixed with 16 units of IS, the minimal amount of IS required to inhibit 64 units of AF, less than 20% of the AF and none of the IS is recovered after chromatography. Apparently the two components remain bound to each other and do not separate on the column. No AF or IS activity is recovered from a mixture of 64 units of AF and 32 units of IS while about half of the IS activity is recovered when 64 units of AF and 64 units of IS are chromatographed together. The binding of IS to AF is saturated at a ratio of one unit IS to two units AF. At $10^{-4}$ *M* $Ca^{++}$ binding did not occur and both AF and IS are fully recovered after chromatography of a mixture.

## *Tissue-specific expression of tubulin RNAs during sea urchin development.* Kristi Wharton, Glenn Merlino, Rudolf Raff, and Joan Ruderman (Marine Biological Laboratory).

We have examined tubulin gene expression in sea urchin embryonic ectoderm and endoderm. Ectodermal cells were dissociated from *Lytechinus pictus* plutei by treatment with an isotonic glycine-EDTA solution and purified by filtration through a 28 μm Nitex mesh. Preparations of endodermal tissues were collected from Triton X-100 treated plutei by differential centrifugation. RNA was isolated from each tissue preparation by a guanidine-HCl extraction, electrophoresed on agarose gels, and transferred to nitrocellulose filters.

*Lytechinus pictus* cDNA clones complementary to α-tubulin (pα2) and to β-tubulin (pβ2) sequences were used as hybridization probes for tubulin mRNAs. Total cellular pluteus RNA contains two β-tubulin RNA transcripts of 1.8 and 2.2 kb in length, which probably represent mature mRNAs, as well as more weakly hybridizing bands of 4.5, 6.5, and 15 kb in size. The α-tubulin message is 1.75 kb in length, and weaker high molecular weight RNAs were found at 2.3, 2.6, 3.8, 4.5, and 15 kb. The levels of both α- and β-tublin mRNAs are considerably higher in ectodermal than in endodermal cells (2–5 fold).

The ectodermal cells of intact sea urchin embryos may be deciliated by a hypotonic seawater shock. Plutei were treated by three rounds of deciliation, each followed by a 90 min recovery time. These deciliated embryos exhibited a 4–6 fold increase in mature tubulin message sequences (β-, 1.8 and 2.2 kb; α-, 1.75 kb) in the ectoderm. In contrast the endodermal cells showed at most a 1.5 fold increase in tubulin sequences. The high molecular weight band sequences show a 3–4 fold increase in response to deciliation. These larger sequences may be nuclear precursors since they are readily detected in nuclear RNA.

The high levels of tubulin mRNAs in ectoderm are consistent with the heavy ciliation of the ectoderm and with the higher level of tubulin proteins observed by 2-D gel analysis of pluteus tissue. As in other systems (*e.g. Chlamydomonas* and *Tetrahymena*) the response of ectodermal cells to deciliation requires augmented synthesis of tubulin mRNAs. Both β-tubulin mRNAs increase in deciliated embryos suggesting that either both encode ciliary tubulin or that deciliation induces a non-specific rise in tubulin synthesis.

This work was supported by NIH grant #HD15351 (to J.V.R.) and the Embryology course at MBL.

### *Lucifer yellow CH as a non-intrusive,* in vivo *fluorescent probe for physiological studies during early development.* R. I. WOODRUFF, D. A. LUTZ, AND S. INOUÉ (Marine Biological Laboratory).

We used the fluorescent dye Lucifer yellow CH for following early developmental events directly in living embryos. This negatively charged dye is freely diffusable through gap junctions for *ca.* 30 minutes, after which it binds to cell constituents; its fluoresence is proportional to concentration (Stewart 1978, *Cell* **14:** 741–759).

We monitored distribution of iontophoretically micro-injected dye by a high sensitivity (SIT) video camera attached to a microscope with crossed polarizers and appropriate filters in the trans-illumination mode. The fluorescent image of dye-injected sea urchin blastomeres was too faint to be detected by the dark-adapted eye but was clearly displayed through the SIT camera. With a video analyzer, we could monitor the rising level of fluorescence injection and graphically display the intensity distribution of the diffusing dye, sharply peaked at the tip of the injection needle. The volcano-shaped distribution converted to an ellipse conforming to the cell shape within seconds, once the injecting current was turned off. The amount of dye rose linearly during iontophoresis, became constant thereafter, and was unaffected by change in cell shape during cleavage.

Individual micromeres were injected with Lucifer yellow at the 16-cell stage and their development followed. Scattered throughout the rings of primary mesenchyme cells, about one-quarter of the cells displayed Lucifer fluorescence; in contrast a quadrant of the archenteron also fluoresced at the gastrula stage. At the prism and pluteus stages, fluorescently labeled offspring of the single injected micromere were seen crawling along the birefringent spicules. The cells divided and developed normally and synchronously with non-injected sister cells.

This non-intrusive technique allows one to trace the fate of individual cells with great precision and shows promise for quantitating the fluorescence in localized regions within living cells.

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## NEUROBIOLOGY

### *Trigonometric nearest neighbor analysis of the neuroplasmic lattice arrays in axons.* W. J. ADELMAN, JR. (Laboratory of Biophysics, NINCDS, MBL), A. J. HODGE, AND R. B. WALTZ.

Electron micrographs of transverse sections of myelinated axons found in sciatic nerves of the toad, *Bufo woodhousi fowlerii,* were examined so as to measure the spatial characteristics of the lattice array of filamentous elements in the axoplasm. Cross-sections through the Schmidt-Lanterman "cleft" regions were chosen for analysis because the axons were invariably constricted in these zones, with a consequently higher packing density of longitudinal filamentous elements and seemingly better preservation of order than that found in internodal regions. The analytical technique involved use of a TV camera to produce a monochrome image of a print on a color terminal linked to a PDP-11/60 computer. Appropriate programming allowed the cursor coordinates to be inserted into a memory file upon command following placing of the cursor over the observed lattice locations of the neurofilaments and/or neurotubules. Insertion of coordinates into memory was confirmed by an "echo subroutine" which generated a bright spot on the screen at the cursor position, thus eliminating duplication of digitized points and allowing direct visualization of the matrix in memory. A subroutine was written to analyze the data. For each memory location, the routine searched for all other locations within a specified vector radius, and thereby generated (a) the vector length distribution, (b) the number of neighbors for each point, and (c) the angles between vectors to nearest neighbors. The program was tested and proven operational using an electron micrograph of a transverse section of blow fly flight muscle in which the myofilaments were located in a known hexagonal array. Analysis of the neuroplasmic lattice in toad axons showed a relative invariance of the angular distribution with increasing vector length as compared with the upward progression of nearest neighbor number. This analysis indicated that the lattice most clearly approximates a hexagonal array.

### *Fast axonal transport in lobster axons.* ROBERT D. ALLEN (Dartmouth College), RAYMOND J. LASEK, SUSAN P. GILBERT, ALAN J. HODGE, AND C. K. GOVIND.

The motor axons to the claw closer muscle of juvenile lobsters, *Homarus americanus,* are valuable for examining fast axonal transport since they contain microtubules but not neurofilaments. Using the

Allen video enhanced contrast (AVEC) system with polarized light (POL) microscopy or differential interference contrast (DIC) microscopy, rapid particulate movements can be seen in a field of 21 μm along any selected region of the axon. Three size categories of organelles are observed to move in both the orthograde and retrograde directions. Mitochondria 2–20 μm (or more) in length undergo interrupted movements in either direction. Some of these movements appear to be elastic recoil. They also perform "acrobatic maneuvers," such as "loop-the-loop" and "snake descending a staircase" movements. Medium size (0.35–1.0 μm) particles, thought to be mostly vesicular elements, exhibit discontinuous movements, and their rates of movement are more rapid. Much greater numbers of small particles (<0.2 μm) the size of synaptic vesicles and other tubulovesicular elements move in the orthograde direction at an average velocity of 3.84 ± 0.88 μm. Fewer particles in this size range move in the retrograde direction slightly more rapidly (4.18 ± 0.95 μm/sec). In most but not all instances, organelles and small particles can be seen to move along longitudinally oriented linear elements which are presumably microtubules since micrographs of those same axons show a network of microtubules with few if any neurofilaments. The apparently smooth, continuous movement of small particles in either the orthograde or retrograde direction is believed to be the fundamental process of fast axonal transport because these particles, unlike the larger organelles, are programmed to move in a single direction. At the light microscope level it is clear that these movements are seen in the vicinity of microtubules. In the lobster, the microtubular-based system seems adequate to support fast axonal transport without invoking a possible role for neurofilaments.

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## *Seasonal changes in the circadian modulation of sensitivity of the* Limulus *lateral eye.* ROBERT B. BARLOW JR.

A circadian clock in the *Limulus* brain generates efferent optic nerve activity at night (*Science* **197**: 86–89, 1977). The efferent activity changes the structure and function of the lateral compound eyes causing a dramatic increase in retinal sensitivity at night (*Science* **210**: 1037–1039, 1980).

I report here that the circadian modulation of retinal sensitivity changes with the time of year. During the summer, circadian rhythms in the amplitude of the ERG exhibit short nights relative to those measured during the winter. In both cases animals were exposed several weeks to the natural light-dark cycle of sunlight and then clamped to a rigid platform in an aquarium located in a light proof, shielded cage. ERG's elicited by dim, brief flashes presented every 15 minutes were recorded with corneal electrodes while the animals remained in the dark. The amplitudes of the ERG's were plotted and measurements were made of the animals "subjective night length", which is defined as the number of hours the ERG amplitude exceeds 25% of the difference between the daytime and nighttime levels. Measurements from 75 *Limuli* yielded subjective night lengths ranging from 9 to 11 h in the summer and from 13 to 15 h in the winter. Intermediate values were recorded in the spring and fall. The seasonal changes in visual responses corresponded reasonably well to the seasonal changes in sunset and sunrise for 43°N latitude. No seasonal changes were detected in the circadian periods.

The circadian clock appears to adapt its duty cycle for generating efferent activity to the seasonal changes in night length. Do the seasonal changes reflect continuous adjustments of the circadian clock to fluctuations in daylight? Or are they generated by an endogenous circannual clock?

I thank Joseph Fladd for technical assistance. Supported by NIH grant EY-00667 and NSF grant BNS 81-19436.

## *Somatotopy within the medullary electrosensory nucleus of the skate,* Raja erinacea. DAVID BODZNICK (Wesleyan Univ., Middletown, CT) AND ANNE W. SCHMIDT.

Ampullae of Lorenzini are electrosensory organs innervated by the anterior lateral line nerve (ALLN) on the head and pectoral fins of elasmobranchs. Anatomical (Koester and Boord 1978, *Am. Zool.* **17**: 431) and physiological studies (Bodznick and Northcutt 1980, *Brain Res.* **195**: 313) have demonstrated that electroreceptor afferents terminate in the medullary dorsal nucleus (DN). We now report that the terminations of these electroreceptive fibers are somatotopically organized.

The electrosensory organs of skates occur in three major groups on each side of the body, innervated by three separate ALLN rami. In 7 animals the proximal cut end of an individual ramus was soaked in 1% lysolecithin nearly saturated with HRP. After 8–16 days the animals were perfused with glutaraldehyde and brain sections examined for peroxidase activity (TMB reaction).

The three ALLN rami innervating clusters of ampullae project to non-overlapping portions of the DN neuropil. The external mandibular ramus that innervates the largest and most caudal hyoid cluster of ampullae terminates in a large dorsal portion of DN. The superficial ophthalmic ramus from the most rostral ampullae on the snout projects to the most ventral portion of DN, and the buccal ramus innervating ampullae on the lateral part of the head terminates in the central portion. These dorso-ventral divisions can be recognized in normal nissl-stained sections as distinct areas separated by compact cell plates.

In single-cell recordings the receptive field maps of DN neurons confirmed this organization. Individual electrode tracks revealed that the most dorsal cells received their input from small numbers of ampullae of the most-caudal hyoid group and cells with buccal or superficial opthalmic inputs were encountered ventrally in DN.

The wide distribution of ampullary organs on the body surface of skates provides a means of localizing electric field sources (*e.g.* prey animals). This spatial information is preserved within the medullary electrosensory nucleus.

This work was made possible by The Grass Foundation and an NIH grant to D.B.

## *Fast axonal transport in isolated axoplasm of* Myxicola infundibulum. ANTHONY C. BREUER (Cleveland Clinic Foundation), PETER A. M. EAGLES, SUSAN P. GILBERT, ROBERT D. ALLEN, JANIS METUZUALS, DAVID F. CLAPIN, AND ROGER D. SLOBODA.

The giant axon of the marine fan worm, *Myxicola infundibulum*, has received considerable attention because of the unusual preponderance of neurofilaments and paucity of microtubules in the axoplasmic cytoskeleton and the ready accessibility of the axoplasm, which can be pulled out of the giant axon from the intact organism in 10 seconds (Gilbert 1972, *Nature New Biol.* **237:** 195–197). We report the visualization of moving organelles in *Myxicola* axoplasm using AVEC-DIC video-enhanced microscopy (Allen *et al.* 1981, *Cell Motility* **1:** 291–302), a Hamamatsu C-1000 Chalnicon camera and Polyprocessor frame memory to subtract out-of-focus background mottle. Specimens collected in England were rinsed in calcium-free sea water and axoplasm was removed, sandwiched between two No. 0 coverglasses, surrounded by 1.01 osmolal glutamate buffer pH 7.0 with 0.5 m*M* ATP and immediately examined with the microscope. Translocation of mitochondria, intermediate sized particles (about 200–300 nm diameter), and small particles (about <100 nm diameter) was readily visualized and persisted for up to 2 hours. Transport was bidirectional for all classes of particles and could be seen throughout the axoplasm, although the preponderance of traffic was noted near the surface. Distinct linear organelles could be seen in some sequences, and elongate mitochondria and smaller organelles could be seen moving along them, at times retracing their progress for more than 20 μm along the same "track." We interpret these motions as rapid axonal transport of membranous organelles along linear elements believed to be microtubules. Ultrastructural analyses are in progress. The small numbers of distinctly visible and spatially separate linear elements seen in the axoplasm by AVEC-DIC microscopy and the vastly fewer translocating organelles relative to squid and lobster axons may make this system simpler to analyze. Further study of isolated axoplasm of the *Myxicola* giant axon may prove useful in unraveling the molecular mechanism of fast nerve cell transport.

## *A relatively robust, single-trial, associative learning in the opisthobranch mollusc,* Pleurobranchaea californica. L. B. COHEN AND J. E. FREEDMAN (Dept. of Physiology, Yale University School of Medicine).

With the aim of developing a preparation with relatively few, large neurons that could be used for studies of the cellular basis of learning, we have carried out behavior experiments on *Pleurobranchaea*. The paradigm we have used is called taste-aversion learning, a subset of the paradigms called classical conditioning.

For each experiment 5–8 animals were divided into two groups. Both groups were tested to determine the concentrations of lobster extract and honey necessary to elicit a criterion response (partially everted proboscis). One to four hours later one of the foods was paired with a 1–3 mg/ml solution of quinine, an aversive substance. After a further delay of 1 to 24 hours, the animals were retested to see if the quinine-pairing affected the concentration of food-substance needed to elicit the criterion response. In our best experiment, the pairing led to a relative decrease in response to the paired food when compared to the control food in each animal. Statistical analysis of the results from this experiment showed that the results could occur by chance with a probability of less than 0.02. Six additional experiments of this kind were done. When the results of all seven were combined, the mean change was in the expected direction and the probability that the result was due to chance was less than 0.001. The mean relative increase in concentration of the paired taste needed to elicit the criterion response was a factor of 3.

While we are hopeful that changes in the protocol can lead to an even more robust and larger behavioral change, we think that the results already obtained are good enough to allow us to begin cellular studies.

Supported by N.I.H. grant NS 08437.

## *Pharmacological properties of isolated and cultured horizontal cells of the skate retina.* JOHN E. DOWLING (Harvard University), ERIC M. LASATER, AND HARRIS RIPPS.

The ability to maintain intact, identifiable nerve cells in culture affords a unique opportunity to study the interaction of neurotransmitter candidates with cell-surface receptors. In the present study, horizontal cells were isolated from the all-rod retina of the skate by treating the retina with papain in Leibovitz's tissue culture medium (L-15), adjusted for isotonicity with skate CSF. The cells were mechanically disassociated by repeated pipetting, plated out in tissue culture dishes containing the modified medium, and maintained in culture for up to three weeks. Although the horizontal cells tended to alter their shapes and to retract their fine processes during the first 24 h in culture, they retained most of their morphological features and began to sprout new processes during the culture period. Prior to determining the pharmacological properties of the cells, the culture medium was replaced by an elasmobranch Ringer's solution.

The results reported here were obtained by intracellular recording from cells maintained in culture for 2–5 days. Immediately after penetration, horizontal cells had resting membrane potentials of −15 mV to −30 mV, and input resistances of 50–70 megohm. Within a few minutes, however, resting potentials usually increased to final values of between −70 and −90 mV, and input resistances reached 150 megohm. Cells with resting potentials greater than −60 mV were tested for their responsiveness to transmitter agents applied via pressure ejection through multi-barreled pipettes. L-glutamate and the glutamate analogs quisqualate and kainate produced depolarizations of up to 90 mV at concentrations (in the delivery pipettes) of less than 100 $\mu M$. No responses to L-aspartate were observed unless 5 m$M$ or more of drug was used. The cells were also highly responsive to $\gamma$-aminobutyric acid (GABA); concentrations of less than 100 $\mu M$ GABA produced long-lasting depolarizations of up to 80 mV that resembled the glutamate responses. The responses to GABA could be partially blocked by bicuculline. Skate horizontal cells in culture were unresponsive to D-glutamate, glycine, D- and L-aspartate, dopamine, carbachol, and serotonin applied at concentrations of 1 m$M$.

This research was supported by grants EY 00824 and EY 00285 from the National Eye Institute, USPHS.

## *Circadian clock generates efferent optic nerve activity in the excised* Limulus *brain.* LESLIE E. EISELE, LEONARD KASS, AND ROBERT B. BARLOW, JR. (Syracuse University, NY).

A circadian clock in *Limulus* brain generates efferent optic nerve activity at night leading to various changes in retinal structure and function (*Science* **197**: 86–89, 1977; *Science* **210**: 1037–1039, 1980). We developed an excised brain preparation to study efferent and afferent connections to this circadian clock. The brain was dissected free from the rest of the animal and placed into a temperature-controlled chamber filled with an organ culture. Glass suction electrodes were positioned along the various desheathed optic nerve stumps. Occasionally we recorded from or electrically stimulated different bundles of the same nerve.

Efferent activity recorded from the lateral optic nerve (LON) *in situ* resembles that recorded from the excised brain in the following ways: the efferent activity which begins in the early evening occurs in discrete bursts, and the general level of activity changes from day to night. Efferent activity persists for up to 3 days in the excised brain. The bursting efferent activity recorded from an LON is synchronous with that recorded from the opposite LON, the median optic nerve (MON), and the ventral eye nerve (VEN). Bisecting the isolated protocerebrum desynchronizes the bursts of efferent activity in opposite LONs. Thus, efferent cell bodies are located in both sides of the protocerebrum. Further lesions of the brain suggest that the location of the cell bodies may be limited to the lamina or medulla.

Electrically stimulating both MONs induces efferent activity recorded from both LONs. Illuminating the excised brain tends to inhibit the efferent activity in LON.

In sum, the excised brain appears to be a viable preparation for further studies on central visual pathways in *Limulus*.

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## *EM and AVEC-DIC analyses of membranous organelle transport in squid giant axons and isolated axoplasm.* M. A. FAHIM, S. T. BRADY, A. HODGE, AND R. J. LASEK (Anatomy Dept., Case Western Reserve Univ., Cleveland, OH).

Recent developments in video enhanced light microscopy (AVEC-DIC) permit visualization of particles moving in both orthograde and retrograde directions in the squid giant axon and isolated axoplasm. The dominant feature in these studies is the presence of vast numbers of small particles and other tubulovesicular elements moving parallel to linear elements in both directions. In order to analyze these particles and relate them to identified cellular organelles, axonal transport was blocked focally by cooling a 3 mm region of the axon to 4°C for 1–4 h. Many more moving particles were observed adjacent to the cold block area. On the proximal side of the cold block large numbers of small particles and other tubulovesicular elements were most frequently seen moving parallel to linear elements. By contrast, medium size vesicles and large membranous bodies were enriched on the retrograde side of the block. Similar results were obtained from intact axons and isolated axoplasm.

Using 4% glutaraldehyde in EGTA-phosphate buffer (1200 mosm, pH 7.2), intact axons and extruded axoplasm were fixed and prepared for EM. After locally cooling the extruded axoplasm orthogradely transported particles accumulated just proximal to the cooled site resulting in a distinct increase in the number of small particles at that site. Electron micrographs revealed that the small particles are mostly tubular and vesicular structures (40–50 nm in diameter) which accumulated in files parallel to the long axis of the axon. Many of the small vesicles were similar in size to synaptic vesicles. The particles accumulating in the retrograde direction tended to be larger (80–100 nm) and included many double membrane structures. Particles accumulating in both directions have a dense granular material associated with the pathways. Microtubules were less frequent in the cooled area, while neurofilaments were apparently unaffected by the cold. These results suggest that different identifiable axonal components travel in different directions along the axon in association with linear pathways.

Supported by a Grass Fellowship to Dr. M. A. Fahim.

### *Membrane changes in a single photoreceptor cause retained associative behavioral changes in* Hermissenda. JOSEPH FARLEY, WILLIAM G. RICHARDS, LORRAINE LING, EMILY LIMAN, AND DANIEL L. ALKON (Section on Neural Systems, Lab. of Biophysics, NINCDS, NIH, MBL).

Previous research with *Hermissenda* has demonstrated striking *correlations* between the associative suppression of phototaxis and biophysical changes intrinsic to two of the three type B photoreceptors. Repeated light-rotation pairings produce cumulative depolarization and probable increase in intracellular $Ca^{++}$ in the type B cells. This results in long-term inactivation of a fast, outward $K^+$ current ($I_A$) in B cells, observable for days following training. We now report that type B cells are *causally* related to associative modification of phototaxis.

Single type B photoreceptors were impaled in restrained animals, and were then exposed to either: 1) five pairings (at 2-min intervals) of 30 sec of light and depolarizing (+15 mV) current, or 2) five unpaired (*i.e.* separated by 30 sec) presentations of light and current (at 2-min intervals). For a third "sham" treatment, intracellular penetration of B cells lasted for less than 5 min. Measurements of changes in membrane potential and resistance were obtained 5 min following training. Animals were then allowed to recover and were subsequently re-tested for phototaxis.

Light-current pairings produced a cumulative depolarization of 5.23 mV (±S.E.M. of 0.79 mV) in B cells ($n = 24$), which was absent for cells exposed to the unpaired treatment ($\Delta V_m = 0.83$ mV ± 0.93; $t(45) = 3.55$, $P < 0.001$). Input resistance was also increased for the paired (31.60 MΩ ± 1.37 to 47.10 MΩ ± 2.25; $t = 2.05$, $P < 0.05$) but not unpaired (38.53 MΩ ± 1.13 to 39.65 MΩ ± 1.69) treatment conditions, by 48%.

For those animals which recovered, "blind" measurement of phototaxic latencies 48 h post-training revealed a pairing-specific suppression of phototaxis. Test latencies were significantly longer for paired (102.00 min ± 7.27; $n = 13$) vs. unpaired (52.65 min ± 21.76; $n = 6$; $t(17) = 2.56$; $P < 0.01$) and vs. sham (67.60 min ± 18.03; $n = 7$; $t(18) = 1.98$, $p < 0.05$) treatments, which did not differ.

### *Kits of voltage-sensitive fluorescent probes for external or iontophoretic staining of central nervous systems or single neurons.* A. GRINVALD (Weizmann Institute of Science), R. HILDESHEIM, J. PINE, AND L. B. COHEN.

Recent experiments on several different preparations indicated that some of the best probes evaluated on squid giant axons are not useful for optical monitoring of neuronal activity in other preparations. However, in such cases, close analogs were often found to give large signals. Therefore we have synthesized 45 analogs of styryl dyes. We designed families of probes whose net charges are either negative, neutral, positive, or doubly positive. The length of the conjugated chain was also varied (two, four, or six carbons). The aliphatic substituents on the anilino nitrogen were varied from a methyl to a hexyl. All of these dyes were tested on squid giant axons, in voltage-clamp experiments. The largest fluorescence signals were obtained with analogs having the dipenthyl-anilino chromophore. However, when these dyes were tested on other preparations they were not uniformly successful. Even though RH-421, the dipentylanilino and sulfobutyl styryl, exhibited a fractional change of 25%/100 mV when tested on neuroblastoma cells maintained in culture, in experiments on *Aplysia* neurons maintained in culture this dye gave small signals. For *Aplysia*, RH-376 (the propyl phosphonate analog of RH-160) had to be used to obtain large signals.

To allow optical measurements of synaptic responses from the site of synapses on dendrites, we have designed the doubly positively charged dyes for iontophoretic injection into single cells. We found that dyes with short alkyl groups (RH-355 and RH-461) on the anilino nitrogen diffused quickly into the processes of injected leech neurons. These dyes are the dimethyl and diethyl analogs and have a trimethylammonium propyl side chain and four carbons in the conjugated chain.

For optical recording of cortical activity in the mammalian brain, many of these dyes were evaluated by testing them on the rat visual cortex, and again many did not perform well. However, RH-292 (a triethyl ammonium propyl, dibutyl anilino styryl) did (see Orbach *et al.* 1982, *Biol. Bull.* **163:** 389).

We conclude that optical monitoring of membrane potential is more likely to succeed if 10 to 30 voltage sensitive dyes rather than a few probes can be evaluated for each given preparation.

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## *Central organization of vestibular efferent neurons in the toad fish,* Opsanus tau.

STEPHEN M. HIGHSTEIN AND ROBERT BAKER (Marine Biological Laboratory).

The semicircular canals, saccule, and lateral line organs are innervated by efferent vestibular neurons whose cell bodies lie in a medial nucleus in the medulla below the cerebellum. Efferent somata were distributed rosto-caudally between two superficial dorsal commissures 200 $\mu$m apart. Coronally they lay in a dorsal subgroup above and a ventral subgroup along the median longitudinal fasiculi. Saccular and lateral line efferents overlapped those of the canals with an additional 10% located 200 $\mu$m behind the more caudal commissure. Saccular efferents comprised most of the dorsal subgroup and canal efferents the ventral. However, a single neuron efferent to any end organ could be in either subdivision. Efferent neurons were always found bilaterally but with an ipsilateral predominance (3 to 1). Each semicircular canal was innervated by 30–40 neurons; saccular efferents numbered 140–150. Dendrites of efferent neurons from the dorsal subgroup interdigitated bilaterally, those located ventrally were exclusively ipsilateral providing evidence for possible separate as well as group recruitment. Axons of canal efferents traveled anteriorally for 250 $\mu$m in a paramedian dorsal trajectory before turning laterally to cross the medullary tegmentum and exit the brainstem. Most saccular efferents pursued the same course but about 10% followed a more ventral trajectory near the median longitudinal fasiculi before they ascended to join the above bundle. Somata of canal efferents were antidromically identified and were penetrated with glass microelectrodes containing horseradish peroxidase. Most canal efferent neurons were only antidromically activated from one peripheral site indicating separate populations of efferents to each canal. Straddling of the antidromic stimulus revealed underlying short latency depolarizations that were shown to be indicative of electrical coupling. Coupling was predominantly limited to neurons from the same canal. Efferents were spontaneously active and discharged with much higher frequency when the fish was roused to movement from any sensory stimulus. The above patterns of efferent physiological activity suggest several roles consistent with putative inhibitory action on hair cells. Activity in the absence of movement indicates a tonic modulatory influence, and their strong recruitment associated with movement, another type of regulatory mechanism.

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## *Correlation of electron microscopic fine structure with videomicroscopic observations in identified lobster axons during glutaraldehyde fixation.*

A. J. HODGE (Laboratory of Biophysics, NINCDS, MBL), C. K. GOVIND, R. J. LASEK, AND R. D. ALLEN.

A preparation containing two excitatory motor axons ($\approx$30 $\mu$m in diameter) from the claw closer muscle of a juvenile lobster was observed by video-enhanced AVEC-POL and AVEC-DIC microscopy. In one of these axons, the usual long mitochondria were present and moving normally. Both orthograde and retrograde transport were also clearly visible against a reasonably well-resolved background of filamentous elements (microtubules) and Brownian movement was not obvious. The neighboring axon, however, contained immobile abnormal appearing mitochondria with blebs, many "particles" showing considerable Brownian movement, and there were no indications of transport. In all likelihood, this axon was "dead." Both axons were located relative to an easily recognizable feature in the surrounding connective tissue, and the axons were then externally irrigated with an isotonic fixative. The net result of this was the cessation of all movement without any detectable change in optical properties, and a decrease in Brownian movement.

The fixed preparation was subjected to routine post-fixation with $OsO_4$, acetone dehydration, and embedding in Epon 812. Relatively thick ($\approx 0.2$ μm) transverse sections were observed in stereo using a Philips EM400 electron microscope. Clear-cut differences between the two axons were seen in transverse sections. One definitely exhibited the "normal structure" already established for lobster and other arthropod axons, *i.e.*, it contained a well-ordered neuroplasmic lattice consisting of neurotubules linked transversely by periodically disposed cross-bridges and the usual complement of organelles and small vesicles. The other axon appeared rather degraded by the same criteria. The neuroplasmic lattice showed considerable deterioration, and numerous vacuoles were present in the axoplasm. The results indicate that the cross-linking activity of glutaraldehyde does very little other than to maintain the structural integrity of the axoplasm, at least insofar as its optical properties (DIC) are concerned. These results are in accord with published x-ray diffraction observations on protein crystals and paracrystalline arrays (myelin sheath) showing that the net effect of glutaraldehyde fixation is the addition of small bridging elements without appreciable loss of order.

## *Organization of mononeuronal pools innervating muscles of the free fin rays in the searobin,* Prionotus carolinus. KATHERINE KALIL (University of Wisconsin) AND THOMAS E. FINGER.

Searobins (*Prionotus carolinus*) possess 3 pairs of fin rays used for exploratory movements. Each fin ray is moved independently by a pair of muscles, an elevator and a depressor. The rostral spinal cord of the searobin has 3 pairs of enlargements of the dorsal horn termed accessory lobes. Previous experiments (Finger 1982, *Biol. Bull.* **163:** 154–161) established that the sensory nerve to each fin ray terminates in a single accessory lobe. These projections are arranged somatotopically such that the ventralmost fin ray is represented within the caudalmost lobe, while the pectoral fin is represented rostral to the lobes. The present experiments were carried out to determine the organization of the motoneuronal pools innervating the fin ray and pectoral fin muscles and the extent to which this pattern corresponds to that of the sensory projections.

Injections of HRP into individual fin ray muscles showed that the motoneuronal pools are discrete for each fin ray and lie ventral to the accessory lobes. The motor pools are arranged in a somatotopic order similar to the sensory projections. That is, the ventralmost fin ray is innervated by the caudalmost motoneurons whereas the pectoral fin motoneurons lie rostral to the accessory lobes. However, motor neurons are not in precise register with the lobes; rather, the motoneuronal pool innervating a given fin ray is shifted forward of the corresponding sensory projection by a distance approximately equal to one half a lobe.

There are no obvious differences in the location, numbers, or sizes of the motoneurons innervating the two different muscles of each fin ray. Moreover, all of the retrogradely labeled neurons lie in the ventral motor cell column. The unlabeled dorsal motoneurons may innervate the epaxial or dorsal fin erector muscles.

These results coupled with previous studies indicate the possibility of a local reflex pathway for each fin ray.

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## *Light-evoked field potentials and* $[K^+]_o$ *in the skate retina: pharmacological studies on the cellular origins of the responses.* C. J. KARWOSKI (University of Georgia), R. L. CHAPPELL, L. M. PROENZA, R. B. SZAMIER, D. J. TAATJES, V. MANCINI, AND H. RIPPS.

There is good evidence that the most prominent electrical potentials that comprise the transretinally recorded electroretinogram (ERG) result from light-induced changes in extracellular potassium $[K^+]_o$ acting passively on membranes of non-neuronal elements. For example, the light-evoked decrease in $[K^+]_o$ recorded in the region of photoreceptor inner segments hyperpolarizes apical membranes of pigment epithelial cells which, in turn, generate the slow vitreous-positive c-wave. A light-evoked increase in $[K^+]_o$ seen more proximally in the retina is thought to give rise to Müller-cell currents resulting in the earlier, more transient b-wave. In addition, it has been suggested that the distal decrease in $[K^+]_o$ acts also on the Müller cell to produce a transretinal potential similar in time course to the c-wave but opposite in sign, *i.e.*, the slow PIII component of the ERG. The results we have obtained using various pharmacological agents and recording with conventional and $K^+$-selective electrodes support the view that slow PIII is a $K^+$-dependent response, but they are not consistent with the notion that it originates across the Müller-cell membrane.

Adding the potent gliotoxin DL-α-aminoadipic acid (α-AAA) to perfusate bathing the skate eyecup severely (and selectively) disrupts the structural integrity of the Müller-cell membrane and disperses its cytoplasmic contents. Although 50 m*M* α-AAA abolished the b-wave, it did not affect the distal decrease in $[K^+]_o$, the c-wave or slow PIII.

The distal decrease in $[K^+]_o$ was also insensitive to 1.0 m$M$ $Ba^{2+}$, which eliminated both the c-wave and slow PIII, leaving intact the b-wave. Since $Ba^{2+}$ exerted no effect on this decrease of $[K^+]_o$, these findings indicate that changes in electrical activity due to $Ba^{2+}$ result from direct action of this agent on pigment epithelial cells and on the (unspecified) cellular generators of slow PIII. On the other hand, the fact that slow PIII and c-wave were suppressed by $Ba^{2+}$, whereas the b-wave was unaffected, suggests that there is a fundamental difference in the mechanisms by which these field potentials are generated, *e.g.* in the nature of the $K^+$-channels of the cells subserving these responses.

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### *Efferent neurotransmission of circadian rhythms in* Limulus *lateral eye: single cell studies.* LEONARD KASS AND ROBERT B. BARLOW, JR. (Syracuse University).

A circadian clock in *Limulus* brain generates efferent lateral optic nerve fibers at night. The efferent fibers terminate in the retina and presumably release one or more neurotransmitters that mediate numerous changes in retinal structure and function (*Science* **197**: 86–89, 1977; *Science* **210**: 1037–1039, 1980).

Long-term recordings from single optic nerve fiber afferents indicate that at night the steady-state response characteristics change in 3 ways: (1) spontaneous spike activity is lowered; (2) quantum catch, or sensitivity, is increased; and (3) gain, or response per photon, is increased (*Science* **197**: 86–89, 1977). We report that octopamine (1 $\mu M$), forskolin (10 $\mu M$), and dibutyrl-cAMP (100 $\mu M$) injected subcorneally into the lateral eye *in situ* during the day induce all 3 changes in the optic nerve response. Intracellular recordings from single photoreceptor cells show that at night the frequency of spontaneous fluctuations in membrane voltage (dark bumps) decrease whereas the response to light increases (*Nature* **286**: 393–395, 1980). Subcorneal injection of octopamine during the day reproduces these changes.

Octopamine has met all five criteria for efferent neurotransmission in *Limulus* lateral eye: synthesis, localization, and release (*Science* **216**: 1250–1252, 1982); physiological mimicry and pharmacological blockade (*Biol. Bull.* **159**: 487, 1980; *Biol. Bull.* **161**: 348, 1981). cAMP may function as a secondary transmitter. Forskoline, a putative adenylate cyclase activator and dibutyrl-cAMP both change retinal structure and physiology in a manner similar to the octopamine-induced changes.

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### *Colchicine blocks nerve excitation: an optical study.* DAVID LANDOWNE (University of Miami), JAMES LARSEN, AND KEVIN TAYLOR.

Internal application of 30 m$M$ colchicine to perfused, voltage-clamped squid axons produced a rapid, specific, and reversible decrease in sodium current to about one-third of control values. The change in axon birefringence which normally occurs when the membrane is depolarized was also dramatically and reversibly decreased by colchicine. The birefringence response to a hyperpolarizing pulse showed only a slight decrease. Application of 10 m$M$ colchicine, had similar but lesser effects on both the electrical and the optical responses. A saturated (less than 10 m$M$) solution of beta-lumicolchicine also had similar effects on both the electrical and optical responses.

Tetrodotoxin, applied externally, completely blocked the sodium current but did not alter the birefringence response either in the presence or absence of colchicine.

The effect of colchicine is to remove or slow an early component of the birefringence response. These experiments clearly demonstrate an association of this component with the sodium conductance change. The site of colchicine action is distinct from that of tetrodotoxin action as seen from their different effects on the optical recordings. The direct involvement of microtubules is unlikely in view of the lumicolchicine results.

Supported by NS137809. We thank I. Llano for sharing her finding that colchicine blocks the sodium current.

### *The carbon fiber electrode: its construction and use in squid axons.* JAMES B. LARSEN (University of Southern Mississippi) AND DAVID LANDOWNE.

Bundles of carbon fibers are a superior alternative to the platinized platinum wire commonly used in voltage-clamp electrodes. Such fibers are uniformly straight, easily manipulated, and rebound without damage after being flexed. Since complex surface preparation is not necessary, carbon fiber electrodes can be assembled quickly with a minimum of experience. Following mild oxidation the current-carrying capacity of fiber bundles is at least equivalent to platinized platinum of equal diameter.

In our electrodes, current is carried by a bundle of 18–20 carbon fibers, each having a diameter of 10 μm (Thornel P-55; Union Carbide Corp.). This is attached with epoxy to the voltage electrode, which is a microcapillary of fused silica containing a fine platinum wire, mounted in the tip of a platinum syringe needle insulated with a polyethylene sleeve. The needle imparts structural strength to the entire electrode assembly and improves the response of the voltage electrode. Electrical contact between a copper wire and the carbon fibers is made with conductive paint containing silver. Spurious current flow from exposed silver and platinum surfaces is prevented with a coating of epoxy. After assembly, electrodes are oxidized in 25 m*M* citric acid, titrated to pH 5.2 with NaOH, for 5 min.

Our experience reveals no change in electrode performance during two months of daily use. Analysis of current traces from typical voltage-clamp experiments suggests that carbon fiber electrodes will support current densities of at least 0.26 mA/cm$^2$ in a 400 μm squid axon, for each fiber included in the bundle.

This work was supported by NIH grant NS137809. We thank A. Strickholm for his kind gift of carbon fibers.

### *Synthesis and release of $^3$H-octopamine from the cardiac ganglion of* Limulus polyphemus. S. C. LUMMIS (Cambridge Univ., UK), P. M. O'CONNOR, AND B. A. BATTELLE.

Octopamine, a biogenic phenolamine, is a likely candidate as a neurotransmitter or neurohormone in the cardiac ganglion of *Limulus polyphemus* (Augustine *et al.* 1982, *J. Neurobiol.* **13**: 61–74). We report that the cardiac ganglion can synthesize octopamine from $^3$H-tyramine and that this newly synthesized octopamine can be released by depolarizing agents. Octopamine in the cardiac ganglion was identified using high voltage paper electrophoresis, and the release of $^3$H-metabolites from isolated ganglion was monitored by liquid scintillation counting. The cardiac ganglion synthesized an average ($n = 3$) of 10.4 picomoles octopamine/mg wet weight tissue when incubated in medium containing 10 μCi $^3$H-tyramine/ml. In addition to $^3$H-octopamine, two unidentified radiolabeled metabolites were detected in the acid extract of the ganglion. The cardiac ganglion released $^3$H-octopamine when stimulated by either 200 m*M* KCl or 50 μ*M* veratridine. The veratridine-induced release was prolonged relative to the KCl-induced release and exhibited a delayed onset of maximum response. In addition, veratridine induced a release of one of the unidentified metabolites. The effects of both KCl and veratridine were blocked by preincubating the ganglion with 40 m*M* $CoCl_2$, suggesting a $Ca^{++}$-dependent release mechanism. The veratridine-induced release was also demonstrated to be $Na^+$ dependent: release was blocked by $Na^+$-free saline. In summary, our results are consistent with the hypothesis of a neuroregulatory role for octopamine in the cardiac ganglion of *Limulus.*

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### *Paracrystalline arrays of neurofilament protein.* JANIS METUZALS, DAVID F. CLAPIN (Faculty of Health Sciences, University of Ottawa, Ottawa KIH 8M5, Ontario, Canada), GLENN J. FENNELLY, AND PETER A. M. EAGLES.

Studies of paracrystalline arrays of cytoskeletal proteins have contributed substantially to the knowledge of the properties of these proteins and their interactions. We are reporting results of experiments on formation of characteristic paracrystalline arrays of neurofilament protein isolated from axoplasm of squid giant nerve fiber. The extruded axoplasm rods were extracted for up to twelve hours at room temperature in the following solution: 300 m*M* potassium methanesulfonate, 150 m*M* taurine, 100 m*M* potassium glutamate, 12.9 m*M* $MgCl_2$, 5 m*M* ATP, 3 m*M* $CaCl_2$, 10 m*M* EGTA, 10 m*M* MOPS, pH 7.2. Analysis of the rods by SDS-PAGE demonstrated the presence of two major peptides (200 and 60K) and a minor band at 223K. SEM and TEM confirmed the presence of 10 nm neurofilaments and their finer, cross-linking structures.

A series of recrystallization experiments was patterned after the procedure used for the preparation of tropomyosin paracrystals. Neurofilament rods (10 to 18) were homogenized in 300 μl of 0.5 *M* KCl, 0.5 *M* $Na_2HPO_4$, pH 7.0 in a chilled glass-teflon homogenizer. The homogenate was dialyzed overnight against 1 liter of 0.05 *M* Tris, pH 8.0 at 4°C. The dialysis was continued against 2 liters of 0.12 *M* $(NH_4)_2SO_4$, 0.01 *M* sodium acetate, pH 5.4 for 10 h at 4°C. Electron microscopy of negatively stained retentate revealed a continuous network of collapsed tubes (diameter 300 nm–1 μm) and rope-like strands (diameter 10 nm–50 nm). The tubes consisted of 2-nm wide unit-filaments intercoiled to comprise 10-nm neurofilaments which are cross-associated into a network. The neurofilaments are oriented at narrow angles against the transverse axis of the tube producing patterns of overlapping striations. Such patterns may result from superposition of helically ordered filaments of the upper and lower wall of the collapsed tube. There is a dark line coinciding with the central axis of many tubes. Analysis of the diffraction pattern of the electron micrographs of the tubes indicated periodicities ranging from 20 to 60 nm. Numerous, randomized, individual 10-nm filaments were also observed.

The observed paracrystalline arrays are expressions of intrinsic properties of the neurofilament protein: phase transition, association in networks and helicity.

This investigation was supported by grant MA-1247 from the Medical Research Council of Canada.

## *Quantitative aspects of growth of an identified neuron in the leech* Hirudo medicinalis. MICHELE MUSACCHIO AND EDUARDO R. MACAGNO (Columbia University).

We have studied quantitatively post-embryonic growth of an identified neuron in the leech using dye-injection and techniques of computer reconstruction. Each segmental ganglion has three pairs of touch sensory neurons (T cells), one with receptive fields on dorsal skin of the corresponding segment. Six dorsal T cells from two adult (three- to four-year-old) animals and six from a juvenile one-year-old were studied. The cells were in ganglia 7 through 10. The lengths of secondary branches, their number and distribution along the main axon, the number of synaptic varicosities, and the total volume of neuropil innervated by the cell were measured. The number of branches varied from cell to cell, as did their average lengths within each cell, by greater than 20%. However, the total length of secondary branching was relatively constant (within 15%) among juvenile and among adult cells. The values of all the parameters measured increased from the juvenile to the adults. The average number of branches increased by 45%, their average length by 50%, and the total length of branching approximately doubled. The number of varicosities increased by 58%, and the volume of neuropil innervated tripled. These changes result in an adult T cell with a lower density of innervation of the neuropil than the juvenile cell.

The data raise several questions about the significance of structural changes during the growth of the T cell. The additional varicosities may represent a strengthening of existing connections or the establishment of new contacts with other post-synaptic targets. New branches may be inserted all along the main processes or only in special regions. Reduced density of innervation by the T cell could reflect increased innervation by other neurons or increased occupation of neuropil by glial processes.

## *Intracellular staining with potentiometric dyes: optical signals from identified leech neurons and their processes.* A. L. OBAID, H. SHIMIZU, AND B. M. SALZBERG (University of Pennsylvania).

Using a fluorescent potentiometric dye, injected iontophoretically, we have been able to record selectively and without signal averaging, optical signals corresponding to action potentials in somata and main processes of identified sensory neurons of the leech, *Hirudo medicinalis*. Lateral P and N cells were filled with a positively charged styryl dye, RH 461 (see Grinvald *et al. Biol. Bull.* **163**: 383) by means of 250 msec, 0.5 nA current pulses (50% duty cycle) applied for five minutes. Following injection, illumination of the ganglion with the green portion (interference filter 540 ± 15 nm) of the output of an electronically shuttered high pressure mercury arc (Osram HBO 100W/2) revealed a strong red fluorescence from the soma and processes. A suction electrode on the ipsilateral posterior root was used to stimulate the injected cell whose main process is directed out the ipsilateral anterior root.

The ganglion was pinned in a chamber mounted on the stage of a modified Reichert Zetopan microscope having focusable stage and head. Epi-illumination with a 40×, 0.75 n.a. water immersion objective (Zeiss) produced a real image above the trinocular tube. A single photodiode (E.G.&G. PV-444) could be positioned behind a set of four independent knife edges, permitting the optical isolation of a region of the preparation of arbitrary size, aspect ratio, and orientation. The fluorescence emission from the injected cell was selected by means of a Zeiss dichroic mirror (FT580) and a Schott glass barrier filter (RG630). Excess noise from the arc was reduced by means of a reference photodiode, located beneath the preparation, which sampled the transmitted intensity. The gain of its photocurrent-to-voltage converter was continuously variable, and its DC output could be matched, by transient nulling, with that of the fluorescence detector. The AC coupled outputs of the two photodetectors were then measured differentially. A response time constant of 600 $\mu$sec and AC coupling time constant of 100 msec were employed.

This technique may be used to study the cable properties of extrasomatic regions of cells, and to monitor the invasion of their branches and large terminals and the integration of information in neuronal arborizations.

We are most grateful to A. Grinvald and R. Hildesheim for the synthesis and gift of RH 461.

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## *Optical monitoring of evoked activity in the visual cortex of the marine rat.* H. S. Orbach (Dept. of Physiology, Yale University), L. B. Cohen, and A. Grinvald.

Recent experiments on the salamander olfactory bulb and goldfish optic tectum showed that optical methods could be used to monitor electrical activity in these preparations. We wanted to determine if the same method would be applicable to the mammalian cortex.

We began by staining tests using 16 fluorescent dyes that were known to give relatively large potential-dependent signals in squid axons to see if they would stain and penetrate into the rat brain when applied in concentrated solutions (0.1–1 mg/ml) to the surface of the cortex. Two pyrazolone-oxonol dyes penetrated 2 mm and four styryl dyes penetrated 200–300 $\mu$m after a one or two hour staining period. These dyes were then tested in optical experiments where visual cortex was exposed and stained; simultaneous measurements of fluorescence were made from 124 cortical loci using epi-illumination and a 124 element diode array on a Leitz Ortholux II microscope. Two kinds of stimulation were used. Either a 20 msec light flash was delivered to the intact eye or the eye was removed and a suction electrode was used to stimulate the optic nerve directly.

Optical signals from the cortex were found in response to both kinds of stimulation. The signals in response to optic nerve stimulation were relatively large, $\Delta F/F$ was $1\text{–}3 \times 10^{-3}$, and reached a peak 20 msec after the stimulus. The signals in response to light stimulation reached a peak about 60 msec after the beginning of the light flash. Measurements at wavelengths outside the absorption band of the dyes did not give rise to signals; thus the signals were not the result of light scattering changes or mechanical artifacts.

Although additional experiments are needed to determine the localization of these signals to specific areas of cortex, our results suggest that optical methods may provide a powerful tool for monitoring activity in many cortical sites simultaneously. We think that such a method could be useful in studying cortical organization.

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## *Asymmetry in the olfactory system of the winter flounder,* Pseudopleuronectes americanus. P. D. Prasada Rao, Thomas E. Finger (Univ. Colorado Medical School), and Wayne L. Silver.

During metamorphosis in the winter flounder, the left eye migrates to the right side of the head so that both eyes come to lie on the upper side of the fish. Although migrated, the eyes maintain symmetry in their projections into the central nervous system (Luckenbill-Edds and Sharma 1977, *J. Comp. Neurol.* **173:** 307–318). However, the olfactory organs do not migrate far from their original position, so in the adult the right olfactory organ is located on the upper side while the left is turned partially towards the substratum. Concomitantly, the upward-facing organ comprises 11 or 12 large lamellae whereas the other organ consists of only 5 or 6 smaller lamellae. Also, the right olfactory nerve is thicker, and the right olfactory bulb approximately three times larger, than their contralateral counterparts.

Horseradish peroxidase was used as a neuronal tracer to compare the central projections of the right and left olfactory bulbs. The overall pattern of olfactory bulb projections into the prosencephalon in the flounder did not differ markedly from that reported in other teleost species. However, the fiber bundle extending from the larger olfactory bulb into the contralateral telencephalon via the anterior commissure is thicker than its counterpart arising from the small olfactory bulb. The terminal fields of the large (right) olfactory bulb in the dorsal, ventral, and posterior areas of both the ipsilateral and contralateral telencephalon are quite extensive. In contrast, the projections of the left olfactory bulb into the ipsilateral telencephalon are less elaborate, and the contralateral terminal fields are relatively sparse.

The more extensive projections of the right olfactory bulb are associated with the greater development of the right olfactory organ. The asymmetric projections of the olfactory bulbs in the adult may be due to postmetamorphic differential growth of the olfactory epithelium and bulbs on the two sides of the flounder.

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## *Does the Schwann cell of* Loligo *act as a potassium electrode? Optical studies using potentiometric probes.* B. M. Salzberg, A. L. Obaid, H. Shimizu, R. K. Orkand, and D. M. Senseman (University of Pennsylvania).

In common with that of glial elements in mammalian systems, the physiology of the Schwann cells that intimately surround the giant axons of squid is poorly understood. In *Loligo*, electrophysiological studies have been limited to measurements of the impedance characteristics of the Schwann layer—the small size and tortuous geometry of the cells precluding transmembrane voltage measurements. In *Sepioteuthis* the cells are 3–5 times thicker, and one laboratory has described a long-lasting *hyper*polarization of the Schwann cell in response to rapid trains of impulses. They attribute this to nicotinic cholinergic transmission from axon to Schwann cell (Villegas 1974, *J. Physiol.* **242**: 647–659). We have attempted to measure electrical events in the Schwann cell by exploiting the linear potentiometric changes in light absorption exhibited by membrane stained with an *impermeant* merocyanine-oxazolone dye, NK 2367. Superfused, the probe should bind to Schwann cell membrane and axolemma, optical signals reflecting voltage changes in both cell membranes. Differences between the optical measurement and an electrode recording should result principally from potential variation in the Schwann cell. Perfused internally, the dye should reach only axolemma, and the optical signal, inverted, should closely resemble an electrode recording. We employed volleys of impulses (250–333 Hz) to raise the potassium concentration in the periaxonal space by about 20 m*M*, and we simultaneously monitored the Frankenhaeuser-Hodgkin effect optically and electrically ($\tau = 3.5$ μsec). When the probe was applied intracellularly, the two signals were superimposable, after scaling. Extracellular staining revealed a striking and consistent difference in the envelopes of the spike undershoots. The optical record was altered in the direction expected if the Schwann cell underwent a very small *hyperpolarization*, assuming that dye bound to glial membrane behaves optically as it does when bound to axolemma. The effect, corrected simply for membrane area, was about 0.7 mV and was not sensitive to curare ($10^{-6}$ *M*). Neither ouabain nor strophanthidin ($10^{-4}$ *M*) reliably altered the result, suggesting that an electrogenic pump is not implicated. Similar experiments on unstained preparations disclosed a very small effect, resembling the difference signal, which depended upon the time integral of the outward current (Cohen *et al.* 1972, *J. Physiol.* **224**: 727–752). While much too small to explain our observations, this signal may be enhanced by the extracellular presence of the dye, in a manner dependent upon the geometry of the Schwann layer and, in particular, the Frankenhaeuser-Hodgkin space.

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## *Responses from spinally innervated chemoreceptors on the free fin rays of the searobin,* Prionotus carolinus. Wayne L. Silver (Monell Chemical Senses Center) and Thomas E. Finger.

The spinally innervated free fin rays of the searobin, *Prionotus*, are sensitive to chemical stimuli despite the absence of taste buds or olfactory receptors. The present research examines the sensitivity of the fin ray chemoreceptors to a variety of compounds. Using conventional electrophysiological techniques, we recorded neural responses from the fin ray nerves in immobilized, artificially respired searobins. Amino acids, because of their abundance in searobins' diet and their extreme effectiveness as olfactory and gustatory stimuli in other fish, were the principal compounds tested.

Responses to mechanical and proprioceptive stimuli were observed in all 36 searobins tested, and the fin rays are apparently extremely sensitive to touch and position. Responses to chemical stimuli were obtained in 21 of 36 fish. Chemical stimuli elicited rapidly adapting responses. Betaine HCl (trimethyl glycine) was the most effective compound tested. The order of effectiveness of other stimulatory compounds at $10^{-2}$ *M* was: dimethyl glycine HCl (DMG) > L-α-ABA > gly > L-ala ≳ L-cysh > L-pro > L-thr > L-ser > L-arg > L-phe. In addition, squid extract (1 g/100 ml artificial sea water) was an extremely effective stimulus. Of the 18 amino acids in squid extract, betaine, gly, L-ala, and L-pro are among the five found in highest concentration (Mackie, 1982 in *Chemoreception in Fishes*, T. J. Hara, ed. pp. 275–291). Compounds tested which did not elicit a response at $10^{-2}$ *M* included L-gln, L-glu, sucrose (1.0 *M*), taurine, trimethylamine oxide, choline chloride, and acetic acid (0.02 *M*). The lowest threshold was to betaine($10^{-5.5}$ M), followed by DMG($10^{-4.0}$ *M*), L-α-ABA($10^{-4.0}$ *M*), L-ala($10^{-3.25}$ *M*), gly($10^{-3.0}$ *M*), L-thr($10^{-3.0}$ *M*), L-ser($10^{-2.0}$ *M*), and L-phe($10^{-2.0}$ *M*).

The results show that chemoreceptors on the free fin rays of searobins respond to relatively low concentrations of certain amino acids. The variety and threshold concentrations of stimulatory amino acids resemble those reported for the taste systems of other marine teleosts (Kiyohara *et al.* 1975, *Bull. Jpn. Soc. Sci. Fish.* **41**: 383–391). These results indicate that the chemoreceptors on the free fin rays of searobins are responsive to compounds particularly prevalent in the animal's natural diet.

Supported by a Grass Foundation Fellowship (W.L.S.) and an NSF grant (T.E.F.).

# PARASITOLOGY AND PATHOLOGY

## *Stage-specific gene expression in* Plasmodium gallinaceum. JAY BANGS, STEVEN ZEICHNER, ROBERT BARKER, RICHARD CARTER, AND DYANN WIRTH (Harvard School of Public Health).

*P. gallinaceum*, an avian malaria, undergoes transformation during sexual differentiation that involves extensive morphological changes in a brief time period. In these experiments we examined the biosynthesis and processing of sexual stage-specific proteins. The gametocytes of *P. gallinaceum* can be induced to exflagellate and differentiate through the sexual cycle as far as the ookinete stage *in vitro*. Blood was obtained from infected chickens, washed, and pulse-labeled *in vitro* with $^{35}$S-methionine under conditions preventing exflagellation. Labeled parasites were washed and divided into two groups. One group was allowed to exflagellate, the other was not. Samples were taken immediately after the pulse, at 1 h and at 5 h of chase, and lysed in 1% Triton X-100. Aliquots of each lysate were analysed for total labeled protein by SDS-PAGE. The remainder of each lysate was divided three ways and immunoprecipitated with either normal rabbit serum, rabbit anti-zygote serum, or rabbit anti-ookinete serum. The immunoprecipitates were analysed on SDS-PAGE and the proteins detected by coomassie blue staining and fluorography. Band patterns in fluorographs of both exflagellated and nonexflagellated pulse chases showed remarkable similarity. Most bands showed no change in intensity during the period of incubation. However, one band of $M_r$ 55K decreased in intensity while one band of $M_r$ 36K increased in intensity in both groups. These findings suggest possible protein processing during the chase period. Both anti-zygote and anti-ookinete sera failed to reveal any significant differences in protein labeling of exflagellated and non-exflagellated parasites at both the 1 h and 5 h chase points. At 1 h anti-zygote serum recognized more proteins than anti-ookinete serum. At 5 h this difference was less evident. Analysis of the immunoprecipitates revealed that zygotes and ookinetes have several common antigens, in addition to ookinete- and zygote-specific antigens. These data indicate that proteins recognized by antisera to zygotes and ookinetes are synthesized and present, prior to exflagellation, in the erythrocytic stages.

## *Surface labeling of* Trypanosoma cruzi. TECIA MARIA ULISSES DE CARVALHO AND MIERCIO PEREIRA (Instituto de Biofisica, Bloco G, Centro de Ciencias da Saude, UFRJ, Rio de Janeiro, Brasil).

Epimastigotes and trypomastigotes, two developmental forms of *Trypanosoma cruzi*, were surface-labeled with $^{125}$I using Iodogen (which catalyzes iodination of tyrosine residues) and iodonapthylazide (INA) which labels membrane proteins embedded in lipid bilayer. Epimastigotes were obtained from liquid cultures and trypomastigotes from fibroblasts infected with *T. cruzi.*

A band of molecular weight 75,000 was present only in epimastigotes, and another of molecular weight 90,000 was detected exclusively in trypomastigotes using the Iodogen technique, whereas bands of molecular weight 45,000 and 35,000 were present in both developmental forms of the trypanosomes.

Using iodonapthylazide reagent, three bands were identified, which showed identical molecular weights in both forms of *T. cruzi*. One of them had a molecular weight of 35,000 and this may be identical with the 35,000 mol wt band labeled by the Iodogen. The other bands were unique and had mol wt of 50,000 and 20,000.

In conclusion, the results indicate that the developmental stages of *T. cruzi* have unique surface proteins as detected by labeling tyrosine residues and conserved proteins as determined by labeling the integral membrane proteins.

## *Control of tubulin gene expression during transformation of* Leishmania *parasites from amastigote to promastigote stages.* MARIE-FRANCE DELAUW, SCOTT LANDFEAR, DIANNE MCMAHON PRATT, AND DYANN WIRTH (Dept. of Tropical Public Health, Harvard School of Public Health, Boston, MA).

*Leishmania* parasites grow inside the macrophages of their mammalian hosts as non-motile intracellular forms called amastigotes. When infected macrophages are ingested by the sandfly vector, the parasites are released from the macrophages and undergo a striking morphological transformation to a motile flagellated extracellular form called the promastigote. K. P. Chang and colleagues at the Rockefeller University have shown that tubulin biosynthesis increases dramatically when amastigotes are transformed to promastigotes *in vitro.*

We are studying the control of expression of the tubulin genes in amastigotes and promastigotes. We have used a genomic clone of the alpha-tubulin gene from *Leishmania enriettii*, isolated in the laboratory of Dr. Dyann Wirth, to probe Southern blots of genomic DNA from amastigotes and promastigotes. The preliminary results suggest that the tubulin genes may increase in copy number upon transformation of amastigotes to promastigotes. We are also using a sea urchin beta-tubulin clone, obtained from Dr. Joan Ruderman, to determine whether the beta-tubulin genes of *Leishmania* are amplified or rearranged upon transformation from amastigotes to promastigotes.

### *A gill disease of* Limulus polyphemus *associated with triclad turbellarid worm infection.* JOSEPH M. GROFF (Laboratory for Marine Animal Health, Marine Biological Laboratory) AND LOUIS LEIBOVITZ.

Severe gill erosions were found to occur on the book gills of wild and captive *Limulus polyphemus*. The pathogenesis of these lesions and their relationship to the triclad turbellarid worms, *Bdelloura candida* and *Syncoelidium pellucidum*, and their cocoons (egg capsules) were investigated.

Ante- and post-mortem examinations were conducted on three groups of *L. polyphemus*: six freshly caught specimens, six specimens held in captivity for six weeks, and six morbid or dead captive specimens maintained in a separate collection for an unknown period of time. Hematological studies were conducted, and blood and gills were cultured for bacteria. Tissues were taken for histopathological study.

Normal gill lamellar structure consisted of a double-walled three-layered chitinous cuticle, two hypodermal cell layers, trabecular cells, and previously undescribed multicellular lamellar pillar corpuscles with their associated pore-like structures; the latter two delineated the vascular channels within the gill lamellae. Cocoons produced a pressure atrophy of the associated and adjacent gill lamellae resulting in degeneration and destruction of the gill cuticle. Subsequent changes within the lamellae included blood loss, degranulation of amebocytes, blood coagulation and occlusion of gill vascular channels. Progressive extension of these lesions resulted in gross gill erosions and perforations. These lesions provided a portal of entry for immature worms and bacteria.

This study documents that cocoons and immature, invasive triclad turbellarids are pathogens of *L. polyphemus*. Prevention, control and eradication of the disease in captive laboratory populations of *L. polyphemus* will be evaluated in future studies.

We wish to thank Ms. Amy Stone for her technical assistance. This study has been supported in part by a grant (No. 1-40-PR01333-01) from the Division of Research Resources, National Institutes of Health.

### *Identification of protective antigens of* Schistosoma mansoni *by Eastern blots using monoclonal antibodies.* R. PAUL JOHNSON AND DON HARN (Harvard Medical School).

We have recently produced monoclonal antibodies against the helminth *Schistosoma mansoni* (D. Harn *et al.*, in preparation). These monoclonals bind to the surface of schistosomula as determined by immunofluorescence and produce a decrease in worm burden (40–70% as compared with controls) when administered to mice prior to challenge with cercariae or schistosomula. Immunoprecipitation and isoelectric focusing blots (developed by D.H.) were used to characterize the antigens recognized by these monoclonals. Soluble egg extracts and 0.5% Triton extracts of schistosomula were resolved in agarose isoelectric focusing gels, transferred to nitrocellulose paper by wicking, quenched, incubated with monoclonal culture supernatants, chronically infected or non-infected mouse sera, and probed with $^{125}$I-rabbit anti-mouse immunoglobulin. Immunoprecipitations were performed with 0.5% Triton extracts (with protease inhibitors) of schistosomula metabolically labeled with $^{35}$S-methionine and Protein A-Sepharose beads precoated with rabbit anti-mouse immunoglobulin. Two monoclonal antibodies recognize an egg antigen with a pI of 4.5 and a schistosomula antigen with a pI of 5.7, both of which are also detected by chronic infection sera. Immunoprecipitation with chronic infection sera reveals antigens with molecular weights of 225,000, 115,000, 85,000, 53,000, 40,000, and 35,000; hybridomas specifically precipitate an antigen of 40,000 molecular weight.

These results demonstrate that isoelectric focusing blots (Eastern blots) may be used to characterize antigens recognized by monoclonal antibodies. The Eastern blot has several advantages over the currently utilized techniques of immunoprecipitation and blotting from sodium dodecyl sulfate polyacrylamide gels (Western blots) in that it does not require radioactive labeling of antigen and that it avoids denaturation of proteins, a process which may destroy the antigenic site recognized by a monoclonal antibody. The existence of antigens common to different stages of the parasite, but of distinct charge, prompts further investigation as to the nature of the antigenic site (protein or sugar determinant) and the comparison of Eastern blot and immunoprecipitation results by two-dimensional O'Farrell gels.

## L. enriettii *α-tubulin is produced* in vivo *by* Escherichia coli. PAMELA LANGER, MICHELE JUNGERY, AND DYANN WIRTH (Harvard University).

The study of parasites at the molecular level is limited by the difficulty of isolation and characterization of parasite proteins. As an initial attempt to get expression of a parasite protein in bacteria, we introduced a molecular hybrid of genomic *L. enriettii* α-tubulin DNA and pBR322 (pLT1 constructed by D. Wirth) into maxicells. We report here that we have been able to detect the synthesis of *Leishmania* α-tubulin in *E. coli.* Lysates of maxicells containing pLT1 or pBR322 were spotted onto nitrocellulose filters and incubated with rabbit anti-*L. enriettii* α-tubulin antibody and $^{125}$I-labeled protein A. Spots of bacteria containing pBR322 showed no reaction with antibody whereas those of an *L. enriettii* extract of bacteria containing the α-tubulin gene showed a strongly positive reaction. The synthesized gene product retained its antigenic properties to the extent that it was able to react with the rabbit anti-*L. enriettii* α-tubulin antibody.

It is not yet known whether the transcription was initiated at the α-tubulin gene promoter or another site such as the pBR322 β-lactamase gene promoter. Further studies are in progress to determine whether the *L. enriettii* α-tubulin gene has a promoter functional in bacteria. Such a promoter could be useful in the construction of cloning vectors for other parasite proteins.

In summary, we have observed the synthesis in bacteria of a parasite protein which retains antigenic properties.

## *A phytomastigophorean infection of embryonating sea hares* Aplysia californica. LOUIS LEIBOVITZ (Laboratory for Marine Animal Health, Marine Biological Laboratory) AND THOMAS R. CAPO.

A specific phytomastigophorean infection of laboratory-cultured embryonating sea hares (*Aplysia californica*) is reported. Earliest microscopically detectable clinical signs were observed on the third day after egg cases were laid. It could, however, be observed at any point of embryonal development after the third until the time of veliger release, usually on the eighth day. Earliest stage of infection was initiated by rupture of clear thin-walled cysts, 20 to 40 μm length or width, discharging small infective round or oval organisms, 3 to 6 μm, with euglenoid-type motility. The liberated organisms actively penetrated the outer wall of the egg case, forming fistulous tracts and becoming attached to the embryos in the compartments of the egg case. The organisms migrated to the yolk tissues within the valve, beginning a period of feeding, growth and reproduction. The resulting mature sausage-shaped trophozoites, 15 to 40 μm, conformed to morphologic description of phytomastigophora. The disease spread through the egg mass by direct extension resulting in embryo erosion, lysis, and ultimate death. Bacteria and ciliates were noted secondarily, in late stages of the disease. If infection occurred early (3 days after egg masses were laid), embryonal mortality was high, often reaching 100 percent. In late infection (after the fifth day), mortality was much lower (10 to 20 percent). Surviving larvae remained carriers of the disease organism. Prevention, control, and eradication of the disease is currently being studied.

We wish to thank Ms. Amy Stone and Susan L. Perritt for technical assistance. This project has been supported, in part, by grants from the Division of Research Resources, National Institutes of Health (1-40-PR01333-01) and the National Institutes of General Medical Science (GM23540-06).

## *A competitive inhibition test for diagnosis of schistomiasis using monoclonal antibodies.* MARTIN PAMMENTER (South African Medical Research Council, Box 17120, Congella 4013, Natal, Rep. South Africa), PAUL JOHNSON, AND DON HARN.

Experiments were designed to test the serodiagnostic potential of a monoclonal antibody which binds to surface membranes of the helminth parasite *Schistosoma mansoni.* The antibody is partially protective in passive transfer experiments and is known to be nonreactive to heterologous (filarial) helminth antigens.

The test was designed as a competitive inhibition of the binding of the monoclonal antibody to *S. mansoni* soluble egg extract by serum from infected persons using a solid phase ELISA. *S. haematobium*-infected sera were obtained from schoolchildren of Kwa Zulu in Southern Africa while control sera were drawn from members of the MBL.

Initial experiments suggest that after pre-incubation with a 1:16 dilution of infected serum there is an appreciable reduction in binding of the monoclonal antibody when compared to controls. This inhibitory activity is TCA-precipitable and can also be precipitated in the range of 20% to 40% saturated ammonium sulphate. The activity can also be at least partially removed by passage of the serum through Protein A-Sepharose.

These results suggest: 1) the reaction is a competitive antibody interaction, 2) the antigen to which the monoclonal antibody is directed is immunogenic in naturally infected people, 3) the antigen is common to *S. mansoni* and *S. haematobium*.

(Martin Pammenter received financial aid from the South Africa Medical Research Council to attend the MBL Biology of Parasitism course.)

### *Comparison of labeled membrane proteins of pathogenic and non-pathogenic South American trypanosomes.* DEBRA ROWSE-EAGLE, CARL A. BOSWELL, TECIA ULISSES DE CARVALHO, AND MIERCIO PEREIRA (Tufts University Medical School).

*Trypanosoma cruzi, T. rangeli* and *T. conorhini* share common triatomine vectors, but *T. cruzi* is pathogenic for man whereas *T. rangeli* and *T. conorhini* are not. These host specificities may be modulated by the presence or absence of unique surface membrane components. The epimastigote stage of these trypanosomatids was grown in liquid culture and surface labeled with $^{125}$I using: 1) Bolton-Hunter reagent, which conjugates to amino groups via an active ester reaction; 2) Iodogen, which labels exposed tyrosine residues; or 3) 5-iodo-1-napthylamine (INA), which labels hydrophobic amino acids and lipids.

Labeling with Iodogen or Bolton-Hunter reagent resulted in electrophoretic patterns which were characteristic for each species of trypanosome. While slight differences of mobility patterns were evident between *T. rangeli* and *T. conorhini*, there were major differences between these non-pathogens and *T. cruzi*.

In another experiment cells were treated with trypsin, then labeled with Iodogen to see if cryptic membrane components were exposed. The band patterns of the non-pathogenic species were altered very little, but major changes occurred with *T. cruzi*. Three bands (80,000, 52,00, and 16,000 daltons) disappeared and were replaced by a single new band (14,400 daltons).

All three species have major membrane proteins that label with INA and migrate on 8–20% polyacrylamide gel electrophoresis at molecular weights of 50,000, 35,000, and 20,000 daltons.

In summary, it appears that membrane proteins accessible to surface labeling vary between species, but integral membrane proteins labeled by INA are highly conserved.

### *Membrane labeling of protective antigens of schistosomula of* Schistosoma mansoni. DAN ZILBERSTEIN, PAUL JOHNSON, MIERCIO PEREIRA AND DON HARN (Harvard Medical School).

The non-permeant ($^{125}$I)-iodogen and N-succinimidyl-3(4 hydroxy,5-($^{125}$I)iodophenyl)-propionate (Bolton-Hunter) and the hydrophobic 5($^{125}$I)-iodonapthyl azide (INA) were used to surface label schistosomula of *Schistosoma mansoni*. Using these three reagents nine iodinated proteins (molecular weights of 14,000, 20,000, 28,000, 35,000, 40,000, 50,000, 80,000, 94,000 and 125,000) were identified by SDS-PAGE. An additional 30,000 molecular weight component was labeled by both INA and Bolton-Hunter reagents. Immunoprecipitates using both chronic mouse sera and monoclonal antibody contained two proteins (40,000 and 94,000 daltons) which have been labeled by both INA and iodogen reagents.

Since INA reagent labels proteins embedded in the lipid bilayer, and since the proteins labeled by this reagent were also labeled by the reagents that label proteins externally exposed in the outer membrane, the INA labeled bands are integral membrane proteins. Furthermore, the monoclonal antibody used in this study was shown to be surface membrane specific. This observation suggests that the antigenic epitope recognized in the antigen is in the extra-membrane portion of these molecules.

## PHOTORECEPTORS

### *Calcium injections increase sensitivity in calcium depleted* Limulus *ventral photoreceptor cells.* S. R. BOLSOVER AND J. E. BROWN (State University of New York at Stony Brook).

Bathing *Limulus* ventral photoreceptors in low calcium sea water ([EGTA] = 10 m*M*, free [Ca] measured to be $2 \times 10^{-6}$ *M*) first increased the light-induced current in 1–2 minutes. After 15 minutes, there was a progressive decline of the light-induced current and: (1) an increase of light intensity produced a much more than linearly proportionate increase of light-induced current; (2) delayed, apparently regenerative currents were induced by long flashes; (3) ionophoretic injection of calcium ions from an intracellular pipette containing Ca/EGTA buffer or $CaCl_2$ increased the light response; (4) ionophoretic injection of EGTA decreased the light response. When cells were returned to ASW ($10^{-2}$ $M$ $Ca_o$) their behavior returned to normal. To monitor changes of cytoplasmic calcium we injected single cells with aequorin. Aequorin luminescence recorded from unilluminated cells fell rapidly in low $Ca_o$. In contrast, the increase of luminescence induced by a bright flash (caused by a light-induced increase of cytoplasmic calcium) did not change significantly during the first several minutes in low $Ca_o$; however, during a prolonged period in low $Ca_o$ the light-induced increase of luminescence declined profoundly. Returning the cells to ASW restored both the resting luminescence and the light-induced increase of luminescence. We conclude that one or more steps in the transduction system in *Limulus* photoreceptors requires intracellular calcium. Bathing in low $Ca_o$ for prolonged periods reduces both the free cytoplasmic calcium and the light-induced release of calcium from internal stores, possibly by depleting these stores. In this condition, we propose tentatively that light induces a delayed rise of cytoplasmic calcium that acts to increase the sensitivity of the cell. This hypothesis can account for both the more than linear stimulus-response relation and the delayed, apparently regenerative light-induced currents recorded in voltage-clamped cells bathed in low $Ca_o$.

Supported by EY-01914 and EY-01915.

## *Nucleotide injection abolishes the discrete waves evoked by vanadate in* Limulus *photoreceptors.* D. WESLEY CORSON AND ALAN FEIN (Marine Biological Laboratory).

In previous studies of *Limulus* ventral photoreceptors we have reported that extracellular application of 5 m$M$ vanadate in low calcium (1 m$M$) artificial sea water can 1) induce the production of discrete waves in the dark and 2) prolong the response to dim flashes of light. The vanadate-induced waves were found to be similar to those normally evoked by light. Intracellular injections of fluoride, molybdate, tungstate, or GTP-$\gamma$-S, a hydrolysis-resistant analog of GTP, have previously been found to have an effect similar to that of vanadate, while previous iontophoretic injections of GTP and ATP did not induce the production of discrete waves or prolong the light response. We now report that injection of either GTP or ATP can temporarily abolish both the discrete waves and the prolongation of the light response evoked by vanadate.

GTP (3 cells) or ATP (5 cells) was pressure injected into ventral photoreceptors from electrodes containing either nucleotide at a concentration of 20 m$M$ along with 80 m$M$ potassium aspartate (pH 7.0). Injection of either of the nucleotides abolishes for a few minutes the discrete waves evoked by vanadate in the dark. Injection of either of the nucleotides also temporarily abolishes the vanadate-induced prolongation of the response to dim, 20-msec flashes but does not appear to alter the response in other ways. Control injections of 80 m$M$ KAsp did not reverse the effects of vanadate in 3 cells. Therefore either the nucleotides or one of their reaction products antagonize both of the effects of vanadate.

Supported by grants from the NIH and the Rowland Foundation.

## *Intracellular injection of ATP can reduce spontaneous discrete wave activity in* Limulus *ventral photoreceptors.* ALAN FEIN AND D. WESLEY CORSON (Marine Biological Laboratory).

Spontaneous activity is commonly observed throughout the nervous system in the absence of any apparent stimulus. In *Limulus* ventral photoreceptors, for example, spontaneous discrete waves of depolarization occur in the dark. These spontaneous waves are very similar to the discrete waves that are evoked by light in the same photoreceptors. In the experiments reported here ATP was injected into ventral photoreceptor cells by applying short duration pressure pulses to the back of intracellular micropipettes containing 20 m$M$ $Na_2ATP$, 100 m$M$ KAsp, pH 7.0. In four cells we found that the rate of spontaneous wave occurrence was reduced following the injection of ATP. For the two most active cells having spontaneous rates greater than 1 per sec the spontaneous rate fell by more than 2-fold following injection. Injection of the KAsp solution alone did not lead to a fall in the rate of spontaneous waves. The ATP induced reduction in spontaneous rate occurred without any apparent change in the efficacy with which light could induce the occurrence of discrete waves in the same cells. We do not know whether the reduction in the rate of spontaneous waves is a direct effect of ATP itself, or whether the ATP enters into a series of reactions, the product of which then leads to a fall in discrete wave rate. The spontaneous waves of ventral photoreceptors appear to arise from a molecule other than rhodopsin, therefore we suggest that the effects reported here do not reflect an effect at the visual pigment.

## *Photoreceptors of freshwater turtles: cell types and visual pigments.* LEO E. LIPETZ (The Ohio State University) AND EDWARD F. MACNICHOL, JR.

Photoreceptors of three species of freshwater turtles were characterized as rod or double or single cone, as having or lacking an oil droplet, and by the droplet's spectral transmission. All three species had rods and six types of cones. The double cone consists of a chief cone with an orange (O) droplet and a dropletless accessory cone. The N droplet has no absorption in the visible spectrum; the C has an absorption peak in the near ultraviolet. The R, O, and Y droplets transmit significantly only at wavelengths greater than about 580, 560, and 525 nm, respectively.

The droplets and visual pigments were measured with a computer-controlled, photon-counting microspectrophotometer. In all three species were found a rod visual pigment and red-, green-, and blue-sensitive cone pigments. In *Chrysemys scripta elegans* for each visual pigment the number of photoreceptors in the average, the mean wavelength of maximum absorption, its standard deviation, the mean optical density, and the mean half-width were: (a) for rod, 10, 519.4 ± 3.6 nm, 0.048, 148 TeraHertz (THz); (b) for red-sensitive, 57, 622.7 ± 4.5 nm, 0.034, 142 THz; (c) for green-sensitive, 24, 521.6 ± 3.1 nm, 0.027, 134 THz; (d) for blue-sensitive, 18, 461.6 ± 5.5 nm, 0.019, 133 THz. For *Chrysemys picta* the corresponding values were: (a) for rods, 13, 521.3 ± 1.1 nm, 0.048, 123 THz; (b) for red-sensitive, 15, 623.6 ± 2.6 nm, 0.032, 127 THz; for green-sensitive, 8, 520.6 ± 2.9 nm, 0.025, 133 THz; (d) for blue-sensitive, 9, 461.1 ± 5.2 nm, 0.022, 133 THz.

For the above two species plus *Chelydra serpentina* the visual pigment was identified in the following total numbers of each type: accessory cones, red, 134; chief cones, red, 156; R-cones, red, 192, and green, 2; Y-cones, green, 183; C-cones, blue, 125; and N-cones, red, 25.

This work was supported by NEI grants EY 03743 and EY 0239905.

## *Evidence for the release of a catalytic agent during the latent period of invertebrate phototransduction.* RICHARD PAYNE AND ALAN FEIN (Marine Biological Laboratory).

Dark-adapted *Limulus* ventral photoreceptors respond to a dim flash with a latency of 100 ms. Steady background illumination decreases the latency of the response to a superimposed flash and greatly reduces the response amplitude. The decrease in latency is thought to be due to the release by the background light of an agent that increases the rate of an early reaction in phototransduction. The aim of the present study is to demonstrate that this agent is released during the latent period of the response of a dark-adapted cell to a bright flash and to determine its radius of diffusion.

We have investigated the response of dark-adapted cells to 10-ms flashes delivered as 10 $\mu$m spots of light. The latency of the response per effectively absorbed photon falls from 100 to 50 ms as the density of effective photons is increased from 1 to 300 per $\mu m^2$. That a density of >1 effective photon per $\mu m^2$ should initiate the decreased latency suggests that the agent responsible is able to diffuse over at least the length of a microvillus during the latent period of the response. Comparison of the latent period of the response to a 10–20 $\mu$m diameter spot with that to a diffuse light suggests an upper limit of approximately 10 $\mu$m for the diffusion radius of the agent.

Intracellular injection of calcium is known to reduce the latency of the response. Calcium has also been shown to be released following illumination. If calcium is the agent responsible for the decrease in latency that we observe, then we predict a significant local release of calcium during the latent period of the response to a bright flash delivered to a dark-adapted cell.

## *Evidence for postnatal morphogenesis of skate rods.* R. BRUCE SZAMIER (Harvard University Medical School), HARRIS RIPPS, AND DOUGLAS TAATJES

Visual function in skates (*Raja erinacea* and *R. oscellata*) is subserved solely by the scotopic (rod) mechanism, and the visual cells of this elasmobranch contain only one type of photopigment, namely rhodopsin. However, electrophysiological studies have demonstrated that the photoreceptors, as well as second- and third-order retinal neurons, are responsive to incremental light flashes presented on background luminance levels that extend well into the photopic range. In addition, our histological sections show a number of small, proximally placed, cone-like elements within the photoreceptor layer, which possibly represent another class of visual cell. Nevertheless, our results suggest otherwise.

Ultrastructural and histochemical studies showed that the membranous discs of the outer segments of these cells were isolated from the plasma membrane, and that their synaptic terminals appeared immature, unlike those usually associated with cone receptors. In addition, the pattern of incorporation of $^3$H-fucose, as revealed by radioautography, was similar for both the rods and the smaller visual cells; *i.e.*, the label was concentrated along the basal discs of the outer segment. When we examined the disc shedding behavior of the visual cells in skates entrained for two weeks or longer to a 12:12 light:dark cycle, enhanced phagocytic activity was seen only following light onset.

The middle portion of the inner nuclear layer of younger animals contained large numbers of undifferentiated cells with dense nuclei and little cytoplasm. These cells decreased in number with age and were occasionally seen in the OPL or ONL. $^3$H-thymidine autoradiography, used to identify proliferating cells, revealed that these undifferentiated cells were post-mitotic and that retinal neurons were being formed by cell division only in a circumferential ring at the outer margin of the retina.

We conclude that the small visual cells are recently differentiated rods, and are growing and being incorporated into the photoreceptor layer of the retina. These rods appear to originate from undifferentiated progenitor cells in the inner nuclear layer which migrate to the outer nuclear layer.

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## PHYSIOLOGY AND BIOPHYSICS

### *Calcium-dependent potassium current in squid presynaptic nerve terminals.* GEORGE AUGUSTINE AND ROGER ECKERT (Dept. of Biology, UCLA).

In a previous study of inactivation of calcium current in squid presynaptic terminals (Augustine *et al.* 1981, *Soc. Neurosci.* Abstr. 7) it was observed that the Cd-sensitive inward current elicited by depolarizing voltage clamp pulses relaxed more rapidly than the Ca conductance, as measured by Ca tail currents. We report here that this discrepancy is due to the presence of a calcium-dependent potassium current, $I_{K(Ca)}$.

Presynaptic terminals of *Loligo pealei* were voltage clamped with the 3-microelectrode method (Llinas *et al.* 1981, *Biophys. J.* **33**: 289). External tetrodotoxin and 3,4-diaminopyridine and internal tetraethylammonium (TEA) were used to minimize currents flowing through sodium and delayed rectifier channels. Under these conditions the inward current elicited by 100–300 msec depolarizations was followed upon repolarization by a slow outward tail current. This slow tail current ($\tau \approx 0.1$ sec) was blocked by Cd, had a reversal potential near $E_K$ (approx. −80 mV), and was sensitive to the extracellular concentration of K ions. Both the slow tail current and relaxation on inward current were greatly reduced by external TEA (25–200 m*M*). These features are characteristic of the Ca-activated K current, $I_{K(Ca)}$, in many cells, and indicate that the early relaxation of inward current seen without extracellular TEA primarily reflects the simultaneous activation of this outward current. Decay of the Ca conductance, determined from tail current measurements, is a slow, exponential process with a time constant of 1.5 to 5.3 s.

Supported by the Muscular Dystrophy Assn. and USPHS NS 8364.

### *Reproductive strategies of bivalve mollusks from deep-sea hydrothermal vents and intertidal sulfide-rich environments.* CARL J. BERG, JR. (Marine Biological Laboratory) AND PHILIP ALATALO.

A comparison of clams (*Calyptogena magnifica*) from deep-sea hydrothermal vent areas (2500 m) with clams (*Codakia orbicularis*) from intertidal, sulfide-rich creeks and turtlegrass beds reveals several similarities. Individuals of both species are thought to derive major portions of their nutrients from chemoautotrophic bacteria within their gills, based upon morphological, histological, isotope, and enzyme analyses. They also become sexually mature at a relatively young age and release gametes over prolonged periods of time. Both species spawn eggs which are enclosed in gelatinous capsules. Eggs of *C. orbicularis* are 108–112 μm in diameter and are surrounded by a 350 μm diameter capsule. Preserved eggs and capsules of *C. magnifica* are larger, measuring approximately 400 μm and 495 μm diameter, respectively. Under laboratory conditions, veliger larvae of *C. orbicularis* hatch from individual capsules in 2–3 days and are planktonic for approximately 12 days. Larvae maintained in either 1 μm filtered sea water, or sea water with suspensions of cultured phytoplankton, undergo metamorphosis 13–16 days after fertilization. Although egg size is greater in *C. magnifica*, we hypothesize that similar development occurs. Lecithotrophic nutrition of these larvae may be supplemented by planktotrophic and/or chemoautotrophic capabilities.

### *Mechanism and function of synchronous flashing in the firefly* Photinus pyralis. JOHN BUCK (National Institutes of Health), FRANK E. HANSON, ELISABETH BUCK, AND JAMES F. CASE.

The flying male emits a single flash about every 6 s at 23°C. The perched female responds about 2 s after each male flash. The male reaches the female via a succession of such alternated signals. After one male initiates dialogue with a female, other males often join, flashing synchronously with the original male. Synchronization occurs when male A's flash impinges on B 1.5 s or less before B is due to flash. B's flash is then triggered prematurely and his flash-timing and -perceiving cycle is reset. In pacemaker resetting in an Oriental species flashing is delayed one full timing cycle (Buck *et al.*, 1981, *J. Comp. Physiol.* **144:** 287). In contrast, the *P. pyralis* flash delay is only 350 ms, about the latency of cephalic electrical stimulation, as if the timing cycle were reset to its end rather than its start.

If A is in dialogue with a female when he resets B, B can then respond directly to A's female and compete on an even footing. In a pair interaction A thus halves his own chances of being accepted by the female. The evolutionary selection of male-male flash-triggering (synchrony) therefore seems puzzling. In larger groups, however, flash synchronization could prove adaptive. If a 6th male joins 5 synchronized suitors he decreases the reproductive prospects of each of the 5 from 1/5 to 1/6 but increases his own prospects (with that female) from zero to 1/6. He gains no individual advantage over any other male but wins a chance to compete for a rarely found prize. During the many random group encounters of one male with others during his lifetime these (strongly advantageous) opportunities to join courtships of other males more than offset the equal number of (weakly disadvantageous) occasions when he inadvertently causes another male to joint his own courtship.

### *A single calcium-mediated process can account for both rapid and slow phases of inactivation exhibited by a single calcium conductance.* ROGER ECKERT, DOUGLAS EWALD, AND JOHN CHAD (Department of Biology, UCLA, Los Angeles).

Cells L2–L6 in *Aplysia californica* were voltage clamped in artificial sea water containing 0.45 m*M* tetrodotoxin, 200 m*M* tetraethylammonium chloride, and 5 m*M* 4-amino-pyridine to isolate the Ca current, $I_{Ca}$. Depolarizations were to 0 mV or less, lasting up to 900 ms. Calcium tail currents measured at $E_K$ were proportional to the inward current at all times, indicating an absence of contamination by K current. Inactivation kinetics, determined from computer fits, were correlated closely with current strength (*i.e.* peak $I_{Ca}$), and were only secondarily influenced by membrane voltage, $V_m$. Thus, when $I_{Ca}$, elicited at 0 mV, was progressively reduced with extracellular $Cd^{2+}$, the inactivation kinetics slowed dramatically, approximately matching those of currents of similar peak $I_{Ca}$ elicited by smaller depolarizations before the Cd block. Inactivation occurred with two exponential phases, a rapid $\tau_{h1}$, and a slower $\tau_{h2}$ asymptotic to a noninactivating component, $I_\infty$. Progressive reduction of $I_{Ca}$ by whatever means resulted in a progressive disproportionate loss of the $\tau_{h1}$ component, and a slowing of $\tau_{h2}$. At small currents only $\tau_{h2}$ remained. Furthermore, injection of EGTA slowed both $\tau_{h1}$ and $\tau_{h2}$, and increased $I_\infty$. These findings indicate that $\tau_{h1}$ and $\tau_{h2}$ both reflect Ca-dependent processes.

These kinetics, along with other features of the calcium current, were simulated by iterative solution of the following equation, based on Hodgkin-Huxley $m^2$ activation kinetics plus Ca-mediated inactivation proportional to intracellular free $Ca^{2+}$:

$$I_{Ca} = [\bar{G}_{Ca}(V_m - E_{Ca})][m_\infty - (m_\infty - m_0)e^{-t/\tau m}]^2 \cdot 1/(1 + K \cdot S)$$

in which K = efficacy of $Ca^{2+}$ in inactivating Ca channels; $S = \int_0^t (1 - B)I_{Ca}\,dt$; and B = probability that free $Ca^{2+}$ at membrane inner surface will be lost to diffusion or buffering. The model simulates the biexponential kinetics of calcium inactivation seen in molluscan neurons, although it contains only a single class of channels and includes no voltage-dependent inactivation. The biexponential kinetics arise from the interplay of $m^2$ activation and Ca-mediated inactivation that is proportional to current-dependent accumulation of $Ca^{2+}$.

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### *Incorporation of a calcium-selective conductance from* Paramecium *cilia in a planar lipid bilayer.* B. E. EHRLICH (Albert Einstein College of Medicine), A. FINKELSTEIN, M. FORTE, C. KUNG.

*Paramecium* is well suited to reconstitution studies of the voltage-dependent $Ca^{++}$ channel for four reasons: 1) the ionic currents have been well studied electrophysiologically, 2) behavioral mutants lacking $Ca^{++}$ currents are available, 3) voltage-dependent $Ca^{++}$ channels exist on the cilia only, and 4) large quantities of ciliary membrane vesicles (CMV) can be prepared. When CMV are incorporated into a planar lipid bilayer in the presence of KCl, very large (100–400 pS), very slow (open tens of seconds), voltage-dependent cation-selective "channels" are seen. We think these records represent the large pores found after EDTA treatment of intact *Paramecium*. Addition of 100 $\mu M$ $Ca^{++}$ to the vesicle-containing bath irreversibly inhibits 75–90% of the membrane conductance. To investigate the properties of the remaining 10–25% of the conductance, incorporation with $Ca^{++}$ as the only permeant cation has been done. When a $Ca^{++}$ gradient is imposed, a current is measured. From biionic potentials, the relative permeability of $Ca^{++}:Sr^{++}:Ba^{++}:Mg^{++}$ is 1:1:0.5:<0.01. Symmetric addition of $K^+$ at 20–100 times the $Ca^{++}$ concentration will shunt the $Ca^{++}$ current. This result suggests that there is a parallel pathway for monovalent cations.

Initial tests with metal blockers show that half the current is inhibited by 1.5 m$M$ cobalt, 0.5 m$M$ cadmium, or 10 $\mu M$ lanthanum. These values are consistent with those from intact preparations. When CMV from pawn mutants (*Paramecium* with ~10% of the $Ca^{++}$ conductance of wild type cells) are incorporated into the bilayer, we see the same degree of background conductance. However, for a given $Ca^{++}$ gradient the $Ca^{++}$ current is one-tenth the current obtained with wild type CMV.

In summary, the reconstituted currents are comparable to *in situ* $Ca^{++}$ currents in ionic selectivity, in degree of block by metals, and by lack of response in one mutant. We are now investigating the voltage-dependence of these currents.

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### *Transduction and voltage-dependent currents of statocyst hair cells in* Hermissenda. JOSEPH FARLEY AND DANIEL L. ALKON (Section on Neural Systems, Lab. of Biophysics, NINCDS, NIH, MBL).

Statocyst hair cells in the mollusk *Hermissenda* process gravitational information. Motile cilia transduce the effects of gravity through active interaction with statoconia, resulting in mechanical deformation of the hair cell membrane at the basal insertion region of the axoneme. Such stimulation produces increased voltage noise and a depolarizing generator potential if sufficiently intense. We have studied the processes of integration and amplification of these sensory signals in hair cell somatic membrane through current-noise analysis of resting potential conductances, and voltage-clamp studies of the voltage-dependent conductances.

Current-noise amplitude was 1–2 orders of magnitude greater for loaded vs unloaded hair cells, and progressively increased with holding potentials more negative than −40 mV. Removal of extracelluar $Na^+$ from the bath provided a clear and reversible decrease in noise amplitude. These observations indicate that $Na^+$ ions contribute greatly to the depolarizing voltage noise in the unclamped cell.

We have identified two voltage-dependent $K^+$ currents in the hair cells. The fast, rapidly inactivating current ($I_A$) is elicited at −30mV, is TEA-resistant, is abolished by 4-aminopyridine (4-AP), and is inactivated by prior depolarization. The slower, sustained $K^+$ current ($I_B$) is selectively reduced by TEA, but not 4-AP. Both currents are calcium regulated. Removal of extracellular $Ca^{++}$ increased $I_A$ by 10–35% in 10 of 11 cells studied; $I_B$ was affected to a much smaller degree.

Despite the fact that the transduction portion of *Hermissenda* photoreceptors and hair cells are derived from quite different membrane types (rhabdomeric and ciliary, respectively), the method of sensory encoding, integration, and transmission of electrical signals is remarkably similar. In both cases, signal detection is accomplished by increases in voltage noise arising from inward $Na^+$ (and also $Ca^{++}$) current which summates to yield a depolarizing generator potential. The $I_A$ and $I_B$ currents appear to be identical, with the former regulated by $Ca^{++}$.

### *Electrochemical, electron spin resonance and spectroscopic measurements of some cytotoxic quinones.* PETER R. C. GASCOYNE, JANE A. MCLAUGHLIN, RONALD PETHIG, AND ALBERT SZENT-GYÖRGYI (Marine Biological Laboratory).

Cosgrove *et al.* (1952, *J. Chem. Soc.* 4821–4823) have isolated methoxy-p-benzoquinone and 2,6-dimethoxy-p-benzoquinone from fermented wheat germ, and Jones *et al.* (1981, *J. Natural Prod.* **44**: 493–494) have found the 2,6-dimethoxyquinone to be cytotoxic.

Studies in this laboratory on mice inoculated with Ehrlich ascites have indicated that the combination of ascorbic acid (AA) with 2,5- or 2,6-dimethoxyquinone exhibits strong cytotoxic properties. The combination of AA with benzoquinone, monomethoxyquinone or 2,3-dimethoxyquinone does not exhibit such cytotoxicity. None of the quinones were found to exhibit such cytotoxicity in the absence of AA. To understand the basic chemical properties that could be responsible for these observations, time-resolved electron spin resonance measurements have been made of the reactions between AA and these various quinones. For the 2,5- and 2,6-dimethoxyquinone, evidence is found for the production of short-lived ascorbate free radicals which are then scavenged by the quinone to form long-lived semiquinone radicals. Such effects were not observed for the monomethoxy-, 2,3-dimethoxy-, and benzo-quinone interactions with AA.

We have determined the electrochemical potentials for the various quinone-hydroquinone redox couples, and the redox potentials (at pH 7.4 and 25°C.) for 2,5- and 2,6-dimethoxyquinone (35.2 and 79.2 mV, respectively) lie close to that for the dehydroascorbate-ascorbate couple (46.6 mV). For methoxyquinone, 2,3-dimethoxyquinone and benzoquinone the corresponding potentials were found to be 164, 185, and 262 mV, respectively. These data, together with spectroscopic and electrochemical titrations, provides support for the viewpoint that the cytotoxic properties of the 2,5- and 2,6-dimethoxyquinone are related to the production of relatively long-lived free radicals as a result of one- rather than of two-electron reductions by the ascorbic acid.

Professor Gabor Fodor kindly prepared the quinones. This work is supported by the National Foundation for Cancer Research.

### *Incorporation of $^{32}$P-phosphate into lipids and proteins by intact squid giant axons.* R. M. GOULD (Inst. for Basic Research, Staten Island), C. A. MANCUSO, P. GALLANT, AND I. TASAKI.

Axon processes, though lacking the capacity for protein translation, contain enzymes both for synthesis and metabolism of lipids and modification of proteins. The localization of these latter activities to axons has been demonstrated biochemically with pure axoplasm, extruded from squid giant axons. We have demonstrated that axoplasm catalyzes the incorporation of $^{32}$P-phosphate into both lipids and proteins. A variety of other potential lipid precursors, including myoinositol, choline, glycerol, serine and glucosamine are also used in phospholipid and/or glycolipid synthesis by extruded axoplasm.

To study the relationship of axonal lipid and protein metabolism to active properties of the axon, we felt it would be necessary to use an intact axon preparation. Intact axons, incubated in sea water containing radioactive precursors, incorporate label into lipids and proteins. However, when the axoplasm and Schwann cell-rich sheath are separated by extrusion of axoplasm and analyzed separately, the sheath always contained several times more labeled lipid and/or protein, independent of the precursor. In contrast, when we injected $^{32}$P-phosphate into the axons, the precursor was avidly retained in the axoplasm and the labeling of axoplasmic lipids and proteins exceeded that of the sheath.

We have studied the incorporation of injected $^{32}$P-phosphate into lipids and proteins of intact giant axons under four conditions, 1) unstimulated (resting), 2) electrically stimulated at 60/sec, 3) TEA in the injection solution, and 4) NaCl in the injection solution. There were significant increases in the incorporation of label into lipids of both axoplasm and sheath (includes both axolemma and Schwann cells) in stimulated, as well as TEA- and NaCl-injected axons compared with unstimulated controls. The increases in axoplasmic labeling were most apparent in the inositol lipids, particularly the polyphosphoinositides. There were some indications that protein phosphorylation was increased by the presence of TEA. These results show that we have a method for studying phospholipid and post-translational protein metabolism in the squid giant axon and that this metabolism (at least with $^{32}$P-phosphate) is responsive to physiological stimuli.

Supported by grant NS 12980 from NIH.

### *Isolation of an extreme clump-forming bacterium.* ROBERT R. HALL (Nantucket High School, Nantucket, MA 02554), H. O. HALVORSON, AND K. KEYNAN.

While flocculation of microorganisms into aggregates is frequently observed in natural environments, the mechanisms regulating flocculation are not always well understood. To study this process we isolated pure cultures of flocculating cells from a waste treatment plant. Microscopic examination of the flocs indicate that they contain a wide diversity of types with a rod-like organism being the predominant type. Two observations provided the basis for its isolation. First the clumps grow rapidly at low temperature in a dilute synthetic medium. Secondly when the clumps are sonicated in EDTA the cells are briefly dispersed yielding motile cells which rapidly clump. Pure cultures were eventually obtained by isolating colonies rich in clumping cells, sonicating these in EDTA, rapidly diluting and plating these on solid medium and finally selecting the small, late-forming colonies arising from single cells. This process was repeated until pure cultures, as judged by microscopy and colony forms, were obtained. The final isolated culture is a gram-negative rod which exists free as a motile organism, then attaches to glass or forms a tightly packed aggregate. Tests with n-heptane show that flocculation may be due to cell surface hydrophobicity.

### *Characterization of a detoxifying enzyme from squid salivary gland by use of Soman, DFP, and manganous ion.* Francis C. G. Hoskin (Illinois Institute of Technology) and Robert D. Prusch.

Although an enzyme that hydrolyzes the cholinesterase inhibitor diisopropyl phosphorofluoridate (DFP) is present in mammalian (*e.g.*, rat) kidney, another DFPase with a different molecular weight, structure, and properties is present in squid (*Loligo pealei*) nerve (see Hoskin and Roush 1982, *Science* **215**: 1255–1257 for earlier references). Squid nerve DFPase detoxifies DFP more rapidly than another organophosphorus compound, ethyl N,N-dimethylphosphoramidocyanidate (Tabun), whereas this order is reversed for mammalian kidney DFPase. This criterion is cumbersome: DFP releases two strong acids whereas Tabun releases one, making the pH-stat method ambiguous; DFP releases fluoride whereas Tabun does not, making the sensitive fluoride electrode method impossible. Parallel research on venomous neurotoxic agents unexpectedly revealed a high level of DFPase in squid posterior salivary gland. We report a new criterion for differentiating the two DFPases, and its application to the squid salivary DFPase. Another organophosphorus compound, 1,2,2,-trimethylpropyl methylphosphonofluoridate (Soman) is hydrolyzed 20–40 times faster than DFP by rat kidney, whereas squid nerve DFPase hydrolyzes DFP 5–10 times faster than Soman, all under comparable conditions. Rat kidney DFPase is stimulated 2- to 3-fold by $4 \times 10^{-4}$ *M* $Mn^{++}$, whereas squid nerve DFPase is unaffected or slightly inhibited. These observations form the basis for distinguishing squid nerve DFPase from mammalian kidney DFPase, the names not being rigorously indicative of enzyme source or substrate. On this basis the DFPase found in squid saliva is identifiable as squid type DFPase. The enzyme is different from the proteinous toxin also found in squid saliva. There is nearly twice as much DFPase in female squid saliva as in male saliva. The enzyme is also present in whole salivary gland. The natural substrate and physiological role for this enzyme, or for the superficially similar enzyme in mammalian kidney, is the subject of continuing research.

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### *Selection and properties of glucose transport mutants of* Vibrio parahaemolyticus. H. L. Kornberg (Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW, U. K.), T. M. Pernack, and D. J. Schnell.

Like *Escherichia coli, Vibrio parahaemolyticus* takes up glucose via the phosphoenolpyruvate-dependent phosphotransferase (PT) system; unlike *E. coli*, it can apparently carry out cation-linked glucose transport since 5 *μM* carbonylcyanide m-chlorophenylhydrazone (CCCP) powerfully inhibits it. In order to determine the physiological role of these systems, mutants resistant to inhibition by non-catabolizable glucose analogs were selected (a) by cycling *V. parahaemolyticus* repeatedly through media containing fructose ± methyl α-D-glucoside; (b) by repeated culture of the *Vibrio* on a mixture of 0.5% peptone, 0.3% yeast extract, and 0.4% methyl α-D-glucoside (Matsumoto *et al.* 1974, *J. Bact.* **119**: 632–634); and (c) by culturing the organism on L-lactate in the presence of 5-thio-D-glucose. Mutants thus selected did not grow on glucose as sole carbon source, did not take up $^{14}C$-labeled glucose or methyl α-D-glucoside and, when rendered permeable with toluene, did not effect the phosphoenolpyruvate-dependent phosphorylation of these hexoses; however, the mutants were unimpaired in the uptake and utilization of mannose, glucoasmine, and fructose. These latter three hexoses are known to share with glucose the PtsM uptake system of *E. coli*, which also effects the uptake of 2-deoxyglucose. Since this glucose analog was taken up by *V. parahaemolyticus* to only a negligible extent, and since the mutants took up and phosphorylated mannose without significant glucose transport, the PtsM system of the *Vibrio* can play at best only a minor role in glucose transport. Similarly, since the mutants that lacked the ability to phosphorylate methyl α-D-glucoside also lacked the ability to take up more than traces of glucose, the cation-linked glucose transport system (if, indeed it is present) cannot be involved in glucose uptake to any major extent.

### *Characterization of D-xylose and D-glucose transport systems in* Spirochaeta aurantia. Cynthia A. Paden, Susan Roberts, and E. P. Greenberg (Cornell University, Ithaca, NY).

Transport of $^{14}C$-D-glucose and $^{14}C$-D-xylose by the gram-negative bacterium, *Spirochaeta aurantia* M1, was investigated. For these studies, cells were suspended in 10 m*M* potassium phosphate buffer, pH 7, to a final density of approximately $2 \times 10^8$ cells/ml. Uptake of both sugars was linear over the 4 to 5 minute duration of the experiments. A kinetic analysis for uptake indicated apparent $K_m$ values for D-xylose and D-glucose of 7 and 4 *μM*, respectively. The apparent $V_{max}$ for D-xylose uptake was 0.25

nmoles/min/$10^8$ cells and the apparent $V_{max}$ for D-glucose uptake was 0.5 nmoles/min/$10^8$ cells. Transport of both substrates was completely inhibited when cells were suspended in 10 m*M* potassium arsenate rather than potassium phosphate. Addition of PMS and ascorbate provided cells in potassium arsenate with a proton motive force, but did not reverse the inhibition of D-xylose or D-glucose uptake. Uptake of both sugars was inhibited by less than 25% in the presence of carbonyl cyanide m-chlorophenylhydrazone (2 $\mu M$), an agent to collapse the proton motive force in *S. aurantia*. An osmotic shock decreased D-glucose uptake from 0.43 to 0.04 nmoles/min/$10^8$ cells and D-xylose uptake from 0.24 to 0.05 nmoles/min/$10^8$ cells. The velocity of D-glucose transport was not decreased in the presence of 1 m*M* D-mannose, D-allose, $\alpha$-methyl-D-glucoside, or 3-O-methyl-D-glucose and was decreased by 15% in the presence of 2 m*M* D-xylose. D-xylose transport was not decreased in the presence of 1 m*M* 2-O-methyl-D-xylose, $\alpha$-methyl-D-xyloside, $\beta$-methyl-D-xyloside, or $\alpha$-methyl-D-glucoside, but was decreased 85% in the presence of 10 $\mu M$ D-glucose. These studies indicate specific uptake systems for D-glucose and D-xylose. Both systems require a high-energy phosphorylated compound for transport rather than a proton motive force. Furthermore, the uptake systems are sensitive to osmotic shock. These features are similar to those of the binding protein-mediated transport systems in other gram-negative bacteria such as *Escherichia coli*.

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## *An endopeptidase inhibitor, similar to vertebrate α-2 macroglobulin, present in the plasma of* Limulus polyphemus. JAMES P. QUIGLEY (Marine Biological Laboratory), PETER B. ARMSTRONG, PAUL GALLANT, FRED R. RICKLES AND WALTER TROLL.

The plasma of vertebrates contains a variety of macromolecular inhibitors of proteolytic enzymes whose function is to bind and inhibit proteases of both endogenous and exogenous origin. One important member of this family of plasma proteins is $\alpha$-2 macroglobulin ($\alpha$2M). This protease inhibitor is effective against a wide spectrum of endopeptidases and acts by forming a complex that shields the active site of the protease from macromolecular substrates but leaves the enzyme free to hydrolyze low molecular weight substrates. The inhibitory activity of $\alpha$2M is sensitive to mild acidification and also methylamine treatment since the complex formed between $\alpha$2M and protease is stabilized by thiol ester bonds.

A potent protease inhibitory activity has now been detected in the hemolymph of the horseshoe crab *Limulus polyphemus* and possesses many of the characteristics of vertebrate $\alpha$2M. Hemolymph was prepared by bleeding pre-chilled crabs under sterile, endotoxin-free conditions. Cells were removed immediately by low speed centrifugation. Hemocyanin was removed from the plasma by centrifugation at 100,000 × *g* for 4 hours. The resulting clear supernatant contained only 2–4% of the plasma protein and most of the protease inhibitory activity. The inhibitory activity in the supernatant was characterized using a number of protease assays including the hydrolysis of $^{14}$C-casein, $^{125}$I-fibrin and arginyl $\rho$ nitroanilide.

The supernatant inhibited the activity of trypsin, chymotrypsin, plasmin and elastase. The inhibitory activity was due to a high molecular protein which was shown to be sensitive to mild acidification and methylamine treatment. The activity of trypsin against low molecular weight substrates was not inhibited by the supernatant, indicating that the active site of the protease remains free.

To our knowledge this represents the first demonstration of a plasma protease inhibitor in *Limulus*. The striking similarity of the inhibitor to vertebrate $\alpha$2M, coupled with the fossil record of *Limulus*, suggests that such protease inhibitors are relatively ancient molecules. The function and pathophysiology of the molecule in the horseshoe crab is now under investigation.

## *Electrogenic $Na^+/K^+$ pump current and flux measurements on voltage-clamped, internally dialyzed squid axons.* R. F. RAKOWSKI AND PAUL DE WEER (Washington University, School of Medicine, St. Louis).

An improved "pump-clamp" technique has been developed for the direct measurement of electrogenic $Na^+/K^+$ pump current and isotopic fluxes in internally dialyzed squid giant axons. A stable, low-noise voltage-clamp circuit is used to maintain the membrane potential to within ±40 $\mu$V. The electrogenic pump current is measured as the change in holding current produced upon addition of ouabain or the reversible cardiotonic steroid dihydrodigitoxigenin ($H_2$DTG) to the sea water bathing the central pool of the experimental chamber. This central pool is isolated from the adjacent end-pools by petroleum jelly seals. The magnitude of the pump current was about 1 $\mu A/cm^2$. The method assumes that the toxins $H_2$DTG and ouabain produce no change of passive membrane conductance or equilibrium potential of any permeant ion. This assumption is validated by the absence of a response to toxin addition when pump operation was stopped by elimination of a required ion or substrate. That is, no change in holding current was produced by toxin addition if $Na^+$, $K^+$, $Mg^{2+}$ or nucleotides were eliminated from the internal and external solutions. The elimination of both internal $Na^+$ and external $K^+$ produced reversal of electrogenic pump current and $Na^+$ flux. Both forward and reverse pump currents were inhibited by membrane hyperpolarization, suggesting that the pumping rate is not necessarily proportional to the thermodynamic driving force. Preliminary data suggest that the stoichiometry of the squid axon $Na^+/K^+$ pump is $2Na^+/1K^+$.

Supported by NIH grants NS-11223, NS-14856, and the Muscular Dystrophy Association of America.

### *Effect of methyl α-D-glucoside on the growth of enteric bacteria: inhibition and escape from inhibition.* D. J. SCHNELL (Department of Life Sciences, University of Nebraska, Lincoln, NE 68508), T. M. PERNACK, AND H. L. KORNBERG.

When cultures of enteric bacteria, grown on fructose, are diluted into fresh fructose medium that also contains methyl α-D-glucoside, further growth is inhibited. However, in medium of low phosphate content (<0.6 m*M*), this stasis is overcome within 2–4 h: the organisms "escape" from inhibition and their subsequent growth is not affected by the glucose analog.

We have measured the uptake of $^{14}C$-labeled carbohydrates by cells grown on a variety of carbohydrates, and also the phosphoenolpyruvate-dependent phosphorylation of these carbohydrates ("PT-activity") by cells rendered permeable with toluene. *Vibrio parahaemolyticus*, like *Escherichia coli*, effects concomitantly the uptake and phosphorylation of methyl α-D-glucoside via the PT-system ($K_m \approx 10$ $\mu M$). Cells that are at the point of "escaping" from inhibition by 2 m*M* methyl α-D-glucoside contain in undiminished activity the PT-components that were present at the time the glucose analog was added; however, they have elaborated an intracellular activity that causes methyl-α-D-[$^{14}C$]glucoside taken up to be rapidly lost again from the cells. It is possible that exposure of the culture to glucose analogs, in media containing low phosphate concentrations, favors induction of an intracellular phosphatase as well as the periplasmic alkaline phosphatase known to be formed.

After "escape," the glucose-specific components of the PT-system cease to be further made during subsequent growth in fructose in the presence of methyl α-D-glucoside, though the fructose PT-activity continues to be synthesized. Since glucose induces the glucose-specific PT system, its repression suggests that methyl-α-D-glucoside phosphate prevents further expression of the appropriate genes.

### *Perfusion of the squid stellate ganglion through its blood supply: implications for morphological and physiological studies of the squid giant synapse.* E. F. STANLEY (Johns Hopkins Medical School) AND W. J. ADELMAN, JR.

The synapse between 2nd and 3rd order giant axons in the squid stellate ganglion has been used to examine many aspects of synaptic transmission. However, one of the difficulties in using this preparation for both physiological and morphological studies is the considerable diffusion barrier between bathing medium and synapse. To circumvent this problem we have perfused the ganglion through its arterial blood supply.

The anterior aorta was cannulated distal to the single branch that bifurcates to supply both stellate ganglia, in squid ranging from 55 to 115 mm mantle length. The aorta was tied off proximal to this branch and the arteries leading to the ganglia were tied off proximal to the right ganglion and just distal to the left ganglion. The left ganglion could now be perfused *in situ* or after removal *in vitro*. Efficacy of perfusion was tested by passing dye or fixative through the ganglion and by testing time taken for $LaCl_3$ to block transmission. Infusion of dye or fixative resulted in a virtually immediate color change of the whole ganglion and the adjoining nerves which was most evident around the cell bodies but was also evident in small vessels within the neuropil. Application of $LaCl_3$ (which is believed to block $Ca^{2+}$ influx into the pre-synaptic terminal and hence transmitter release) to the bath blocked transmission in 38 minutes, confirming the high diffusion barrier. Infusion of the same $LaCl_3$ solution blocked transmission in 33 or 63 seconds (two experiments). We conclude that perfusion through the blood supply greatly improves access of substances to the giant synapse.

### *Comparative microbiology of metal surfaces in sea water.* MARIANNE WALCH, PAUL J. BOYLE, AND RALPH MITCHELL (Laboratory of Microbial Ecology, Division of Applied Sciences, Harvard University, Cambridge, MA 02138).

Metal surfaces in aquatic systems are sites of intense microbial activity, which can result in the enhancement of corrosion processes. We conducted experiments to understand the processes involved in the attachment of various marine bacteria to specific metals and alloys commonly used in ocean engineering applications. Four types of metal—316 stainless steel, titanium, 90-10 copper-nickel (CA 706), and aluminum bronze D (CA 614)—were exposed both to pure cultures of marine bacteria and, *in situ*, to sea water in Eel Pond at Woods Hole, Massachusetts. After exposure for varying time periods the metals were removed and examined using acridine orange direct counts and scanning electron microscopy.

Present data indicate that both qualitative and quantitative differences in the attached microbial communities occur in response to different types of metal surfaces. In general, the two copper alloys supported smaller and less active populations of bacteria than did similarly treated stainless steel and titanium. Removal of the protective oxide film from a metal surface by acid pickling or sanding appeared to alter bacterial attachment and growth, at least in the short term. Also, bacteria grew more rapidly and reached higher surface populations when metals were exposed previously to high concentrations of dissolved organic matter. Dramatic differences in bacterial attachment behavior were seen between experiments run in artificial sea water, in natural sea water *in vitro*, and in natural sea water *in situ*, emphasizing the need to examine further the effect of environment on microbial attachment to metals.

This research was supported by Office of Naval Research contract number N00014-81-K-0624. The technical assistance of Susan Wolff is gratefully acknowledged.

### *Effects of $H_2O_2$ on the dogfish (*Mustelus canis*) ocular lens.* SEYMOUR ZIGMAN, TERESA PAXHIA, BLENDA ANTONELLIS, AND WILLIAM WALDRON (University of Rochester School of Medicine, Rochester, NY 14642).

High levels of the strong oxidant $H_2O_2$ ($10^{-6}$–$10^{-4}$ *M*) were found in human aqueous humors by Garner and Spector (1981). In this study, fresh dogfish lenses were incubated in elasmobranch Ringer's media plus ascorbic acid at 2.4 mg/ml and concentrations of $H_2O_2$ from $10^{-1}$ *M* to $10^{-6}$ *M* for up to 72 h at 20–22°C. In some experiments, they were also exposed to a long wavelength UV emitting lamp (5 $mW/cm^2$ at 365 nm), with suitable dark controls in parallel. Incubated lenses developed cortical opalescence in 4 h and dense opacity in 17 h due to $H_2O_2$ at $10^{-4}$ *M* (minimal) to $10^{-1}$ *M* (maximal) concentrations. Histological examination revealed no structural defects in the outer cortex or lens epithelium. Lenses did not swell, nor was $^{86}Rb$ or $^{14}C$-$\alpha$-amino isobutyric acid uptake inhibited by $10^{-2}$ *M* $H_2O_2$ for 44 h. Starch iodide and dichlorophenyl indophenol assays showed only 1 and 4% of the $[H_2O_2]$ in the medium was present in the lens cortex and epithelium. Proteins of incubated lenses were extracted and separated by homogenization, centrifugation, and polyacrylamide gel electrophoresis analysis. Aggregation via -SS- bonds was found to be stimulated by $H_2O_2$ at $10^{-3}$ *M*, but not at $10^{-5}$ *M*. Additional near-UV light exposure enhanced aggregation; catalase and DTT inhibited it strongly. Optical spectroscopy of lens proteins and free tryptophan showed that $10^{-4}$ *M* $H_2O_2$ plus near-UV light destroyed their 280 nm absorption and stimulated fluorescence with excitation at 360 nm and emission at 440 nm. Such emissions are found in the proteins of aging, near-UV exposed, and brown cataractous human lenses. Thus, if $[H_2O_2]$ in aqueous humor is $\geq 10^{-4}$ *M*, lens proteins may be aggregated to form lens opacities, but uptake of water, salt, and amino acids is not altered appreciably.

Support: NEI; RPB; Mullie Fund; Pledger Fund.

*Continued from Cover Two*

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*Continued on Cover Three*

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# INVERTEBRATE CELL VOLUME CONTROL MECHANISMS: A COORDINATED USE OF INTRACELLULAR AMINO ACIDS AND INORGANIC IONS AS OSMOTIC SOLUTE

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## ABSTRACT

All cells have some capacity for cell volume regulation when confronted with a hypoosmotic stress. The basis of this physiological response is an extrusion of intracellular osmotic solute. The cells of euryhaline osmoconforming invertebrates are capable of regulating volume over a wide range of external osmotic concentrations. Most of the existing data indicate that these cells utilize free amino acids from a substantial intracellular pool as the solute source. However, recent studies indicate that these invertebrate cells utilize inorganic ions as osmotic solute as well. The relative contribution of each solute type varies from species to species and, perhaps, from cell type to cell type. The two solute types are regulated by different mechanisms and often with different time courses, but both solute control systems function in a coordinated manner to regulate cell volume. In addition, evidence is appearing demonstrating a role for organic solutes in the volume regulatory processes of vertebrate cells. At present, it seems that the volume regulatory mechanisms utilized by all cells may be more similar than currently thought, differing in relative contributions of the two solute types rather than kind of solute utilized.

## INTRODUCTION

The literature reporting on studies of osmotic control amongst the invertebrates is vast. The water balance mechanisms utilized by invertebrate species have been under some form of investigation for all of this century and even earlier. In company with most trends in research of biological function, water balance studies have proceeded from analysis of whole organism responses to the intricacies of cellular physiology. The studies cited in the following pages do not constitute an encyclopedic review, but rather point out some of the more recently discovered features of invertebrate cellular water balance systems and, where there are some data, the similarities of the cellular osmotic control mechanisms between species, the presence or absence of a backbone notwithstanding. My remarks here are confined only to the responses to hypoosmotic stress since that has been the most intensely studied.

### *Whole animal responses — some generalities*

It is now apparent that few, if any, invertebrates are isosmotic with their environment. Even the body fluids of marine osmoconformers are slightly hyperosmotic to their environment (Remmert, 1969; Pierce, 1970; Oglesby, 1978, 1981, for examples). Thus, most, if not all, animals have an osmotic gradient between the environment and the extracellular fluid and some physiological capacity for handling

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the water movement resulting from that gradient. This form of osmotic stress is usually a modest one in an osmoconformer and, as Oglesby points out in his review (1981), easily handled by excretory systems. The osmoregulators may have much larger osmotic gradients between extracellular fluids and the environment, but in the adapted state the water movements are dealt with by appropriate ionic transport systems of excretory systems, gills, guts, and integument. Of greater physiological consequence are changes in the osmotic concentration of the external environment, for example an alteration in salinity, or the occurrence of some pathology causing malfunction of the extracellular osmotic and ionic homeostatic mechanisms. The physiological response by invertebrates to salinity change has been studied in great detail. Pathological studies have been done only in higher vertebrates (see Pollock and Arieff, 1980, for a review) for the most part, and that aspect will be touched on only briefly below.

No animal appropriately tested behaves like a piece of dialysis tubing when exposed to a hypoosmotic stress. The capacity for volume control under such a stress may be limited and the range of tolerated osmotic concentrations narrow, but some volume regulatory capacity is present nonetheless. Osmoconforming animals rapidly swell in response to the osmotic influx of water produced by the hypoosmotic

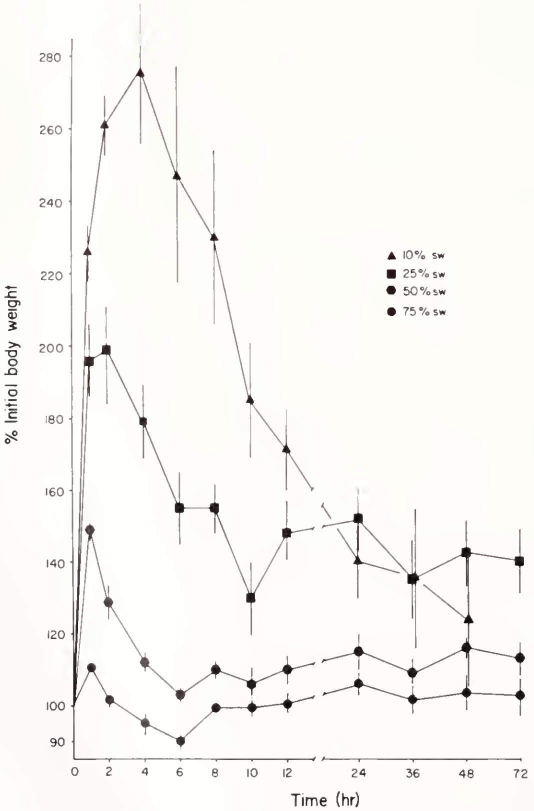


FIGURE 1. The pattern of whole animal volume regulation in *Elysia chlorotica*. At time 0, animals acclimated to 100% sea water were transferred to the salinities indicated and weighed at intervals for 3 days. Each point is a mean from 6 animals. Error bars indicate S.E.M. (From Pierce *et al.*, 1982).

stress, but with time in the reduced salinity will at least partially recover the original volume (Fig. 1). The recovery time course varies from species to species. In general, the more euryhaline an animal the more rapid the recovery. Osmoregulators may show a similar response or may simply swell less than predicted (see Oglesby, 1981, for a thorough review). These whole animal responses are the result of water balance mechanisms which function at two levels within an organism. First, the extracellular systems mentioned above, bulk movement of extracellular water by the excretory system, ion transport by various epithelia, and integumental water permeability all function in some combination to remove the excess water (again, see Oglesby, 1981). In addition, the osmotic influx of water into the extracellular compartments results, perforce, in a dilution of the extracellular environment. This dilution places an osmotic stress on the cells. Thus, although it can not be distinguished by whole animal measurements, the second level of response is at each cell.

All cells tested to date have some volume regulatory ability. Like the whole animal, when the isolated cell is exposed to a hypoosmotic stress, it swells. With time in the reduced osmotic concentration the cell returns toward its original volume (Fig. 2a, b). Few cells, if any, are able to recover the exact original volume. Rather, an incomplete volume regulation is the rule. The cells of euryhaline osmoconforming invertebrates, often naturally exposed several times daily to wide and rapid osmotic fluctuations, are excellent volume regulators, but the cells from invertebrate species have no monopoly on this response. Vertebrate cells also regulate volume albeit usually over comparatively narrow ranges of osmotic concentration (mammalian

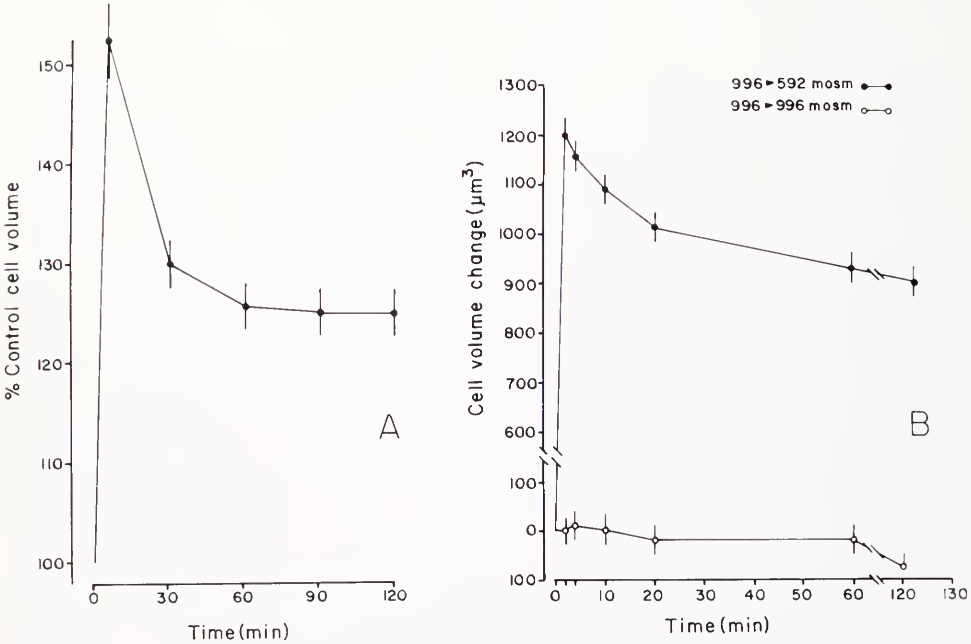


FIGURE 2. (A) Pattern of volume regulation by red blood cells isolated from *Noetia ponderosa* adapted to full strength sea water and exposed to 50% sea water at time 0. Cell volume was determined as packed cell hematocrits. (Data from Amende and Pierce, 1980). (B) Pattern of volume regulation by red coelomocytes isolated from *Glycera dibranchiata* adapted to 996 mosm and then exposed to the osmotic concentration indicated at time 0. Cell volume was measured with a Coulter counter (Data from Costa *et al.*, 1980).

and avian red blood cells [Kregenow, 1971; Poznansky and Solomon, 1972; Schmidt and McManus, 1974], Ehrlich ascites cells [Hendil and Hoffman, 1974; Hoffman, 1978], mammalian heart [Thurston *et al.*, 1981], mammalian brain [Pollock and Arieff, 1980; Thurston *et al.*, 1980], flounder red cells [Fugelli, 1967; Cala, 1977], *Amphiuma* blood cells [Cala, 1980], human lymphocytes [Bui and Wiley, 1981], and rat liver [van Rossum and Russo, 1981] have all been looked at in this regard). No doubt a major reason for the weaker volume regulatory ability of these types of cells is the evolution of vertebrate homeostatic mechanisms.

The volume regulating capacity of the cells of euryhaline osmoconforming invertebrates has attracted some experimental attention from investigators with one of two points of view over the past decade. First, from the point of view of environmental physiology, these cells have been utilized in various attempts to understand the basis of salinity tolerance. Second, from the standpoint of cellular physiology, the mechanisms of cell volume regulation should be more obvious in a cell type that functions over a wide range of osmotic concentrations since the responses should be magnified. The utility of this last approach is only of value if all cells use a mechanism of hydration control in common, at least in its general characteristics. As the studies of invertebrate cell volume regulation unfolded, similarities with vertebrate cell mechanisms were indeed found, but also some major differences. More recent data indicate that the mechanisms may be more similar than we first thought.

### *Invertebrate cell volume regulation in response to hypoosmotic stress — the early results*

The general features of cell volume regulation are the same regardless of the specific source of the cell type. A reduction in external osmotic concentration produces cellular swelling due to osmotic influx of water. To counter the swelling and prevent osmotic lysis, the cells expel osmotic solute together with osmotically obligated water and cell volume recovers back toward, but usually not reaching, the original level. Historically, the osmotic solute source utilized during this process by marine invertebrate cells was presented as being small organic molecules, usually free amino acids, occasionally quaternary ammonium compounds. Vertebrate cells (more likely, terrestrial animals) or cells from freshwater invertebrates, on the other hand, usually seemed to use inorganic ions as osmotic solute. Indeed there is considerable evidence in support of this dichotomy. Each species seems to have its own unique extracellular osmotic concentration, but in general the bloods of marine animals are very close to sea water in osmotic concentration (900–1000 mosm), while the fluids of terrestrial and freshwater animals are much lower in concentration. The osmotic equilibrium between the cells of these organisms and the respective extracellular fluids is such that the osmotic gradient is minimized (although not zero). Thus, the cells of marine invertebrates presumably have osmotic concentrations approximately that of sea water, while the intracellular osmotic concentrations of terrestrial and freshwater animals are much lower. The total intracellular inorganic ion composition of vertebrates seems to account for 60–70% of the intracellular osmotic concentration (see the reviews of Conway, 1957, and Burton, 1968, and the data in Prosser, 1973). On the other hand, in marine invertebrates the intracellular inorganic ion composition is only slightly higher than vertebrate levels (again, see Prosser's [1973] tables). Thus, the total inorganic concentration inside invertebrate cells is much lower than extracellular concentrations. The physiological reason for this ionic discrepancy is not clear. There seems to be some deleterious sensitivity of some enzymes to salt concentrations higher than those found inside cells of animals adapted to sea water (Clark and Zounes, 1977; Bowlus and Somero, 1979; Yancey *et al.*, 1982), but the data are limited at present. In any case, the osmotic differential between the extracellular and intracellular fluids in marine invertebrates is made up by intracellular free amino acids. Furthermore, there are many studies demonstrating that these free amino acids are utilized as osmotic solute during salinity stress (see the reviews by Holden, 1962 for access to the early literature, more recently, Gilles, 1978; Pierce and Amende, 1981).

The intracellular amino acid concentration in a euryhaline invertebrate adapted to sea water can easily be 700–800 m*M* (see for example Pierce, 1971; Costa *et al.*, 1980). The amino acid pool size alone does not always imply salinity tolerance. For example, the ascoglossan opthistobranch *Elysia chlorotica* which has the widest salinity tolerance yet discovered for an osmoconformer (24–2480 mosm) has a tiny amino acid pool (30 $\mu$moles/gm dry wt. in sea water) (Pierce *et al.*, 1982). Still, in most cell types a specific portion of the amino acid pool size declines drastically with acclimation to a reduced salinity. The amino acids utilized vary from cell type to cell type and from species to species but always seem to be some combination of non-essential amino acids. Glycine, alanine, proline, glutamate, taurine, occasionally aspartate and glutamine are the usual amino acids involved (reviewed by Gilles, 1978; Pierce and Amende, 1981).

### *Amino acid mediated volume regulatory mechanisms — older studies*

Various aspects of amino acid mediated volume regulation have been studied in a variety of species and cell types. Of these, one of the more persistent investigations into the mechanisms involved in the regulatory process has been accomplished using two molluscan tissues as model systems: the isolated myocardium of the ribbed mussel, *Modiolus demissus*, and the red blood cell of the blood clam, *Noetia ponderosa*. The results of these investigations have indicated that in response to low salinity, cell volume regulation is accomplished by an efflux of specific amino acids from the cell (Pierce and Greenberg, 1972, 1973, 1976; Amende and Pierce, 1980). The entire decrease in intracellular amino acid concentration is accounted for by the efflux. Thus, there is little, if any, intracellular amino acid catabolism nor protein synthesis which occurs as part of the volume regulatory event. There is some evidence that the amino acids may be catabolized after release from the cells. This is reflected by increases in both blood ammonia concentrations and external ammonia excretion rates (for example Bartberger and Pierce, 1976; Mangum *et al.*, 1976) which follow the appearance of a pulse of amino acids following, in turn, an external salinity decrease (Bartberger and Pierce, 1976). There is also some evidence that the amino acids once released from the cells are sequestered in blood proteins for future osmotic uses (Gilles, 1977; Boone and Schoffeniels, 1979; Péqueux *et al.*, 1979) although this may be a phenomenon peculiar to the arthropods.

The amino acid efflux is initiated by the osmotic pressure change rather than the concomitant external ionic concentration decrease. On the other hand, the reestablishment of normal membrane permeability to amino acids (hence, the magnitude and duration of the efflux) is dependent upon external divalent cation concentrations, ATP concentration, and temperatures and is independent of monovalent cation concentrations (Pierce and Greenberg, 1973, 1976; Watts and Pierce, 1978a; Amende and Pierce, 1980; Otto and Pierce, 1981b). Thus, both an ionic and metabolic component of the efflux control mechanism have been demonstrated. Further, the *M. demissus* myocardial sarcolemma contains substantial divalent cation requiring adenosine triphosphatase (ATPase) activity (Watts and Pierce, 1978b). Inhibition or potentiation of this ATPase activity produced a correlative potentiation or inhibition respectively of the amino acid efflux from the intact heart (Watts and Pierce, 1978c). These results led to the hypothesis that the physiological basis of cell volume regulation, and thereby of low salinity tolerance, in osmoconforming marine invertebrates rests with a membrane bound divalent ATPase which controls amino acid permeability over a wide range of external divalent ion concentrations (Pierce and Greenberg, 1973; Watts and Pierce, 1978c; Amende and Pierce, 1980; Pierce and Amende, 1981). Although no other invertebrate cell type had been studied in this detail up to that point, these results were generally confirmed by others (Gilles and Péqueux, 1981; Pierce and Amende, 1981). At present there are still no data establishing cause and effect between the divalent ATPase and amino acid efflux control, only correlations are established. Furthermore the mechanism of ATPase action is unknown, but most hypotheses suggest a chemo-mechanical system of permeability control such as that found by earlier studies of mammalian cells (Wins and Schoffeniels, 1966; Bowler and Duncan, 1967; Rosenthal *et al.*, 1970; Palek *et al.*, 1971; Rorive and Kleinzeller, 1972; Quist and Roufogalis, 1976).

As the evaluation of these ideas for generality began, some important results appeared. First, a comparison of the two molluscan cellular responses to hypoosmotic stress indeed indicates similar characteristics (ATP and divalent cation requirements for example), but also some interesting differences. The amino acid efflux from the *M. demissus* myocardial cells involves only certain of the many available intracellular amino acids. In these cells the permeability change seems to be quite specific. In contrast, the efflux from the *N. ponderosa* blood cells is similar in composition to the intracellular pool. The significance of this difference between the two species is not clear although it presents the possibility that extremely euryhaline animals (such as *M. demissus*) are so as a result of a highly selective permeability system allowing for both specific solute efflux and intracellular solute conservation. Results with cell types from other euryhaline animals (for example, red coelomocytes from the polychaete *Glycera dibranchiata* [Costa *et al.*, 1980], *M. demissus* myocardium [Pierce and Greenberg, 1972], *Rangia cuneata* myocardium [Otto and Pierce, 1981a]) indicate a selectively permeable volume control system. Second, and of greater importance, is that recent studies clearly indicate the involvement of inorganic ions in cell volume regulation by invertebrate cell types. In addition, in some extremely euryhaline species this ionic component plays a major role in the regulation. The majority of studies which have produced these results have been done on invertebrate neurons.

In spite of the intensity with which nervous function has been investigated, there are surprisingly few data on the effects of osmotic variation on neurons. The volume regulatory response of axons to hypoosmotic stress seems to be quite similar to that found in other cell types (see above). For example, isolated *Callinectes* axons swell during exposure to a hypoosmotic stress and then return toward the initial volume utilizing a mechanism that requires $Ca^{2+}$ and ATP (Gerard, 1975). Axons from other euryhaline crustacea also show similar patterns of volume changes (Gilles, 1973; Kévers *et al.*, 1979a). Although electrical recordings have not accompanied the above studies, neuronal cell volume regulation is accompanied by an adaptation in the electrical properties of the cells. Usually a rapid hyperpolarization of the membrane followed by a slower depolarization and reduction in excitability occurs following a hypoosmotic stress (*Maia* axons [Pichon and Treherne, 1976], *Sabella* giant axons [Treherne and Pichon, 1978], *Mercierella* axons [Benson and Treherne, 1978a, b; Skaer *et al.*, 1978], *Mytilus* cerebro-visceral connective [Willmer, 1978], *Mya* cell bodies [Beres and Pierce, 1979, 1981]).

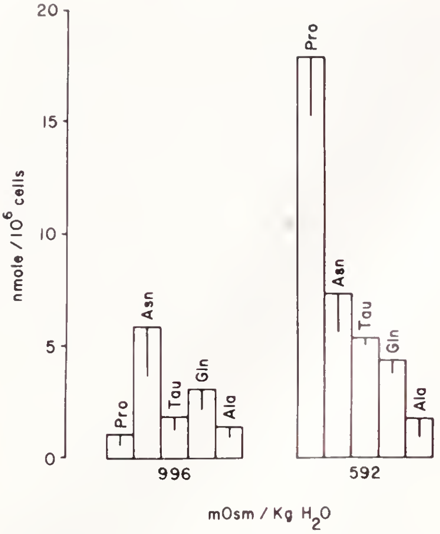
Many of these studies were conducted over short time courses. Recordings made for longer intervals after the salinity decrease indicated that the depolarization and loss of excitability is transient, the time course depending upon the magnitude of the salinity change and presumably the time course of volume regulation. For example, the spontaneous burst frequency, spike pattern within the burst, and resting potential of follower cells in the isolated *Limulus* cardiac ganglion all returned to control levels within 2–3 hours following a salinity change from 100% sea water (SW) to 50% SW (Prior and Pierce, 1981). Similar results occurred with the cell bodies of neurons in the visceral ganglion of the bivalve of *Mya arenaria* (Beres and Pierce, 1981) and the salivary burstor neuron in *Limax* (Prior, 1981). Finally, all of these responses are due to the osmotic rather than ionic change that accompanies the sea water dilution. None of the electrical changes occur if only the ionic concentration is reduced (osmolality maintained with sucrose) (Beres and Pierce, 1981; Prior, 1981; Prior and Pierce, 1981).

### *Invertebrate neurons — cell volume regulation mediated by inorganic solutes*

Only a few studies have examined neurons in this connection. Blue crab axons (*Callinectes sapidus*) volume regulate during hypoosmotic stress using intracellular amino acids from a substantial amino acid pool (Gerard, 1975; Gerard and Gilles, 1972). Osmotic adaptation of other neurons involves at least a partial role of inorganic ions as osmotic solute. For example, the hypoosmotic adaptation of *Sabella penicillus* axons includes a loss of intracellular $K^+$ (estimated from resting potential changes in response to external $K^+$ variation) (Treherne and Pichon, 1978). Treherne (1980) has proposed that the $K^+$ is lost as osmotic solute. Somewhat similar ionic responses occur during the adaptation of both *Mytilus* cerebro-visceral connective axons (Willmer, 1978) and *Mercierella enigmatica* giant axons (Benson and Treherne, 1978b) to hypoosmotic stress. In these two cases, however, the ionic changes alone cannot account for the entire adaptation (Treherne, 1980), and *Mytilus*, at least, has a substantial free amino acid pool (amino acids have not been measured in *Mercierella*). Finally, axons isolated from *Carcinus* lose $Na^+$, $K^+$, and $Cl^-$ at least transiently during volume regulation to a hypoosmotic stress (Kévers *et al.*, 1979b). *Carcinus* axons also have a substantial intracellular amino acid pool (Evans, 1973) which is apparently utilized during volume regulation (Kévers *et al.*, 1979a). Taken together, these studies indicate that amino acid regulation is not the entire story to invertebrate cell volume regulation. There are two other invertebrate cell types that have been studied in some detail with respect to inorganic solute utilization during volume control: red coelomocytes from the blood worm *Glycera dibranchiata* and the isolated myocardium from *Limulus polyphemus*. Both cell types have produced some intriguing results.

### *Glycera red coelomocytes — volume control by amino acids and $K^+$*

The isolated, hemoglobin-containing coelomocytes of *Glycera* rapidly volume regulate in response to a hypoosmotic stress (Costa *et al.*, 1980) (Fig. 2b). The amino acid pool size is large and decreases in content as the cells volume regulate. Furthermore, volume regulation by the isolated coelomocytes is accompanied by an efflux of free amino acids from the cells (Fig. 3) (Costa *et al.*, 1980). The volume regulatory process in these cells requires the presence of extracellular divalent cations but is not specific; either $Ca^{2+}$ or $Mg^{2+}$ will suffice. The volume regulatory process appears to also be dependent upon the metabolic production of ATP (Costa and Pierce, 1982). None of these results is particularly surprising based on previous



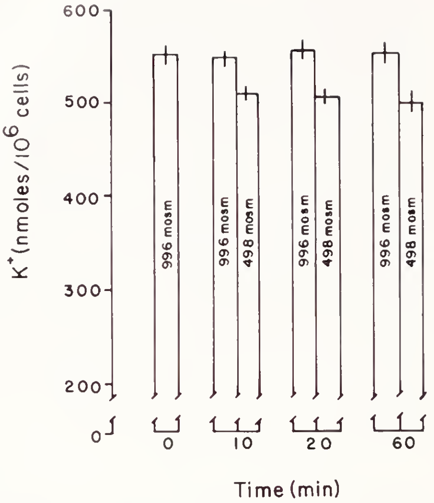


FIGURE 3. Amino acid effluxes from *Glycera* red coelomocytes isolated from worms adapted to 996 mosm and exposed to the osmotic concentration indicated for 40 min (From Costa *et al.*, 1980).

FIGURE 4. Intracellular K$^+$ content in *Glycera* red coelomocytes isolated from worms adapted to 996 mosm and then exposed to 996 or 498 mosm at time 0 (From Costa and Pierce, 1982).

studies, but intracellular K$^+$ content also changes during volume control in these cells.

A rapid decrease in intracellular K$^+$ content occurred in hypoosmotically stressed *Glycera* coelomocytes. Within 10 min of exposure to dilute media intracellular K$^+$ content declined by 10% (Fig. 4). Further, incubation of the cells in Ca$^{2+}$- Mg$^{2+}$-free media appears to disrupt cellular control of K$^+$ content as well as volume regulation. Coelomocytes incubated in Ca$^{2+}$- Mg$^{2+}$-free media lose K$^+$ steadily regardless of the external osmotic concentration. The K$^+$ changes observed were unaffected by ouabain and could be potentiated by incubation in 2-4 dinitrophenol (DNP). This last result is of particular interest because in *Glycera* cells cellular amino acid content was unaffected by DNP not only indicating that K$^+$ and the amino acids leave the cells by different means, but also that the two types of solute are responding to at least some factors not held in common. Finally, the use of K$^+$ as an osmotic solute seems to be only transitory. While intracellular amino acid content is markedly reduced in coelomocytes taken from low salinity adapted worms, K$^+$ content in these coelomocytes is not different from that in cells taken from high salinity adapted animals (Costa and Pierce, 1982) (Table I).

### *Limulus myocardial cell volume regulation — Na$^+$, Cl$^-$, and glycine betaine*

The remarkable euryhalinity of *Limulus* suggests an abundant amino acid pool. However, while the intracellular free amino acid pool of *Limulus* declines with adaptation to low salinity, it is only a small amino acid pool (total = 100 μmole/gm dry wt. in 100% SW adapted crabs) (Robertson, 1970; Warren and Pierce, 1982). Furthermore, amino acids efflux from that pool in response to a salinity decrease, but the efflux is much too small to account for volume regulation (Prior and Pierce, 1981). Instead *Limulus* can tolerate a wide osmotic concentration range without a

TABLE I

*The intracellular* $K^+$ *content of red coelomocytes taken from* Glycera dibranchiata *acclimated to various salinities.*

| Acclimation osmotic concentration (mosm/Kg $H_2O$) | $K^+$ content** |
|---|---|
| 1000 | 417 (±24.8)* |
| 750 | 456 (±22.7) |
| 500 | 413 (±23.7) |

* nmoles/$10^6$ cells (±S.E.).

** Intracellular $K^+$ content is the same for all treatments, and thus the use of $K^+$ as osmotic solute in these cells is only transient (from Costa and Pierce, 1982).

large amino acid pool because the cells regulate volume with a mechanism that relies on inorganic ions and the quaternary ammonium compound, glycine betaine.

The role of quaternary ammonium compounds as osmotic solute was occasionally pointed out in the older literature (for example, Bricteux-Grégoire *et al.*, 1964). More recent investigators have tended to ignore these potentially important compounds largely because their identification and quantification was difficult and rather imprecise. Recently a high performance liquid chromatographic analysis has been developed which solves these analytical problems (Warren and Pierce, 1982). The major quaternary ammonium compounds in *Limulus* cardiac tissue are glycine betaine and homarine. Of these glycine betaine is quite high in concentration in tissue taken from *Limulus* adapted to full strength sea water and declines in concentration in *Limulus* adapted to lower salinities (Fig. 5) (Warren and Pierce, 1982).

The isolated *Limulus* heart volume regulates in response to a hypoosmotic stress. The tissue shows a pattern of incomplete volume recovery quite typical of the pattern exhibited by most cell types (Fig. 6). However, no betaine appeared in the media surrounding the volume regulating hearts and, indeed the betaine content in the tissue was unchanged (Table II) (Warren and Pierce, 1982). Volume regulation by the isolated heart was accomplished without utilizing this major osmotic solute.

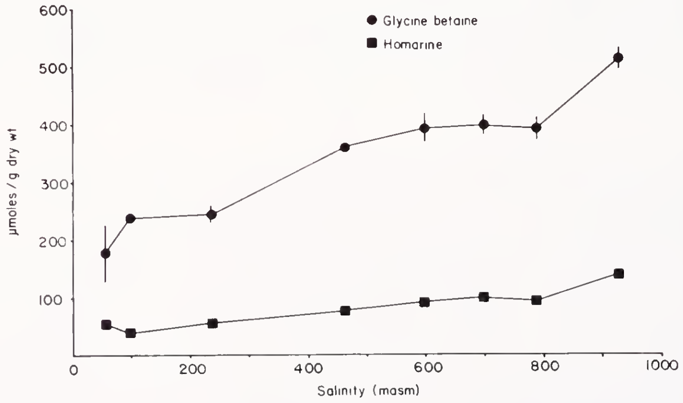


FIGURE 5. Concentrations of the quaternary ammonium compounds glycine betaine and homarine in cardiac tissue of *Limulus* adapted for at least two weeks to the salinities indicated (From Warren and Pierce, 1982).

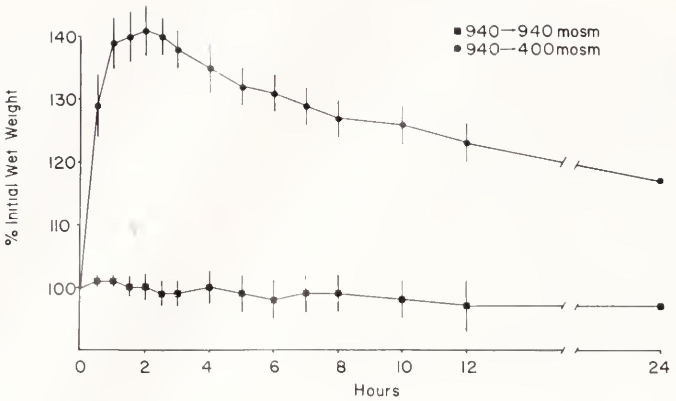


FIGURE 6. Volume regulation by isolated *Limulus* hearts. The hearts were removed from crabs adapted to 940 mosm and then exposed directly to either 940 or 400 mosm at time 0. The hearts were weighed at the time points indicated (From Warren and Pierce, 1982).

Similarly, $K^+$ concentration of the isolated cardiac tissue changed only as predicted by hydration changes (Table III). On the other hand, cellular $Na^+$ and $Cl^-$ levels decreased far more than cell hydration changes could account for during the hypoosmotic stress (Table IV and V) (Warren and Pierce, 1982). Furthermore, these ionic changes are ouabain independent. These results clearly show that $Na^+$ and $Cl^-$ are utilized to regulate volume early on by the *Limulus* cells and glycine betaine much later. Indeed, evidence from whole animal experiments indicates that $Na^+$ and $Cl^-$ partially return toward initial concentrations as glycine betaine declines in the *Limulus* heart cells (Warren and Pierce, 1982).

### *Volume regulation may result from coordination of permeability control systems — conclusions*

The results of the *Glycera* and *Limulus* studies taken together indicate that two quite distinct solute permeability control mechanisms are utilized by these cells during volume regulation. The ions involved ($Na^+$, $K^+$, or $Cl^-$ depending upon the cell type) respond to the decrease in external ionic concentrations which accompany the salinity decrease. The amino acid efflux is triggered by the osmotic change. The ionic movements are not affected by ouabain indicating that the $Na^+$ pump is not

TABLE II

*Glycine betaine concentrations in hearts isolated from* Limulus *adapted to 940 mosm and exposed to 400 mosm.*

| | 940 mosm | 400 mosm |
|---|---|---|
| 6 h | 599 ± 24* | 633 ± 15 |
| 12 h | 621 ± 16 | 631 ± 27 |
| 24 h | 585 ± 21 | 620 ± 21 |

* mmoles/g dry wt ± S.E.

The low salinity values are not significantly different from the high salinity controls (from Warren and Pierce, 1982).

TABLE III

*Intracellular* $K^+$ *in hearts isolated from* Limulus *adapted to 940 mosm and exposed to 400 mosm.*

| Salinity | mmoles/kg $H_2O$ 940 mosm | mmoles/kg $H_2O$ 400 mosm | mmoles/kg $H_2O$ Predicted* | mmoles/kg dry wt 940 mosm | mmoles/kg dry wt 400 mosm |
|---|---|---|---|---|---|
| 6 h | 112.6 ± 7.5 | 74.7 ± 2.9 | 75.0 ± 5.2 | 458 ± 29 | 432 ± 14 |
| 12 h | 113.0 ± 5.7 | 83.2 ± 3.2 | 73.6 ± 4.3 | 432 ± 28 | 453 ± 11 |
| 24 h | 114.5 ± 5.3 | 88.6 ± 3.5 | 79.3 ± 4.6 | 458 ± 20 | 511 ± 13 |

* Calculated according to Freel *et al.*, 1973.

The data are presented in two ways. First, as concentration (mmoles/kg $H_2O$). A concentration decrease of $K^+$ does occur, but only as much as is predicted by changes in tissue hydration. Second, as content (mmoles/kg dry wt). There is no significant change in $K^+$ content indicating that $K^+$ is not used as osmotic solute (from Warren and Pierce, 1982).

involved in the process. It is clear from the data cited above that the two types of permeability systems can be made to operate independently of one another and that they often function with very different time courses in the cell. Nonetheless, it is also clear that both solute control systems operate in concert to control cell volume. The mechanism underlying this remarkable coordination is unknown at present, but may be $Ca^{2+}$ related. The amino acid efflux control mechanism requires $Ca^{2+}$ (see above), and normal $K^+$ permeability in the *Glycera* cells is lost if $Ca^{2+}$ is removed (Costa and Pierce, 1982). However, at present little else is known about the characteristics of the ionic regulatory systems.

Finally, some comparisons to volume regulatory systems found in vertebrate cells may be instructive. There is no doubt that a substantial inorganic ionic component is responsible for volume regulation in vertebrate cells. Usually $Na^+$ or $K^+$ or both are utilized in a ouabain insensitive volume regulatory process that occurs following an osmotic alteration (as opposed to steady state osmotic balance which is usually ouabain sensitive) (reviewed by Rorive and Gilles, 1979). Occasionally other ions are involved. For example, *Necturus* gall bladder epithelial cells require bicarbonate for volume regulation (Fisher *et al.*, 1981). In addition, although there are not yet a lot of data, it seems that vertebrate cells also have an organic solute component to the cell volume regulatory mechanism. This component utilizes amino acids or quaternary ammonium compounds and is particularly obvious in

TABLE IV

*Intracellular* $Na^+$ *in hearts isolated from* Limulus *adapted to 940 mosm and exposed to 400 mosm.*

| Salinity | mmoles/kg $H_2O$ 940 mosm | mmoles/kg $H_2O$ 400 mosm | mmoles/kg $H_2O$ Predicted* | mmoles/kg dry wt 940 mosm | mmoles/kg dry wt 400 mosm |
|---|---|---|---|---|---|
| 6 h | 237.7 ± 13.0 | 79.0 ± 16.9 | 153.1 ± 8.9 | 913 ± 51 | 437 ± 89 |
| 12 h | 228.9 ± 17.6 | 46.3 ± 6.3 | 144.5 ± 10.6 | 905 ± 72 | 273 ± 34 |

* Calculated according to Freel *et al.*, 1973.

These data are also presented two ways (see Table III). There is a substantial change in $Na^+$ concentration which is greater than that which can be accounted for by hydration changes. This is verified by the $Na^+$ content data which also shows very significant decreases during hypoosmotic stress. Therefore, $Na^+$ is used as osmotic solute (from Warren and Pierce, 1982).

TABLE V

*Intracellular $Cl^-$ in hearts isolated from* Limulus *adapted to 940 mosm and exposed to 400 mosm.*

| | mmoles/kg $H_2O$ | | | mmoles/kg dry wt | |
|---|---|---|---|---|---|
| Salinity | 940 mosm | 400 mosm | Predicted* | 940 mosm | 400 mosm |
| 6 h | 199.9 ± 16.0 | 60.3 ± 8.7 | 124.7 ± 9.4 | 780 ± 52 | 352 ± 56 |
| 12 h | 195.8 ± 13.5 | 39.5 ± 4.6 | 135.8 ± 12.8 | 762 ± 48 | 221 ± 26 |
| 24 h | 201.6 ± 15.3 | 38.8 ± 5.1 | 141.0 ± 8.3 | 834 ± 76 | 213 ± 24 |

* Calculated according to Freel *et al.*, 1973.

$Cl^-$ concentration decreased much more than hydration changes could account for and $Cl^-$ content also showed large, significant decreases. Therefore $Cl^-$, like $Na^+$ (Table IV), is regulated in the heart cells in response to hypoosmotic stress (from Warren and Pierce, 1982).

vertebrate species that spend all or part of their lives in water (for example marine toad [*Bufo viridis*] skeletal muscle [Gordon, 1965], flounder [*Pleuronectes flesus*] red cells [Fugelli, 1967], *Myxine* muscle cells [Cholette and Gagnon, 1973], skate [*Raja erinacea*] and stingray [*Dasyatis sabina*] tissues [Boyd *et al.*, 1977], skate [*Raja erinacea*] erythrocytes and muscle [Goldstein, 1981]). Other studies have demonstrated utilization of organic osmotic solute, primarily taurine, in higher terrestrial vertebrates including humans. For example, intracellular taurine concentrations respond to the plasma osmolality changes that occur during hypo- or hypernatremia in both mammalian brain and heart cells (Thurston *et al.*, 1980, 1981; also reviewed by Pollock and Arieff, 1980). Ehrlich ascites cells also utilize taurine for volume control (Hendil and Hoffman, 1974; Hoffman, 1978). At present little is known about the mechanisms utilized to control the organic solutes in these cell types. Nonetheless, the historic intracellular osmotic solute differences held to occur between vertebrates and invertebrates may be a strawman. There is a growing body of information indicating that both types of solute are utilized by all cells, and differences are in magnitude rather than kind. If this turns out to be true, then the cells of euryhaline invertebrates may become important as well as interesting models of osmotic function as a consequence of their remakable abilities of cell hydration control.

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# EFFECTS OF ENZYMATIC AND NONENZYMATIC PROTEINS ON *ARBACIA* SPERMATOZOA: REACTIVATION OF AGED SPERM AND THE INDUCTION OF POLYSPERMY*

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## ABSTRACT

*Arbacia* sperm become inactive after dilution in sea water. We have shown that any of six proteins reactivated the aged sperm as judged by their fertilizing capacity or their motility. In suspensions of inactive sperm in which the mean fertilizing capacity was less than 3%, brief incubation with any of the proteins at 0.5 mg/ml stimulated fertilizing capacity to 70–90%. Reactivation by the proteins was detected at concentrations lower than 2 $\mu$g/ml. All six of the proteins also stimulated motility of aged sperm by 30–70%.

The normal block to polyspermy may involve inactivation of sperm by substances released from the eggs during the fertilization reaction. All six proteins tested on inactive sperm were also shown to induce polyspermy in mixtures of eggs and fresh sperm. Whereas in control mixtures with polyspermic cleavage of ~1% of eggs, proteins at 2 mg/ml induced 5–50% polyspermic cleavage, and induction of polyspermy was detected at 5 $\mu$g/ml.

The six proteins showing activity included enzymes and also the relatively inert gelatin. The concentration dependence was upon weight/volume and not molarity. Though the mode of action is unclear, it must be rather nonspecific, and is certainly not dependent on enzymatic activity. The same mode of action is likely for activation of aged sperm and induction of polyspermy.

## INTRODUCTION

Sea urchin sperm suspended in sea water for a few hours become immotile and lose their ability to fertilize eggs (Gemmill, 1900; Gray, 1928a; Rothschild and Tyler, 1954; Bishop, 1962; Branham, 1966; Mann, 1964; Nelson, 1967). Sir James Gray (1928a) entertained the possibility that this "senescence" might be reversible. Although inactivation was ascribed to a "loss of energy reserves" (Gemmill, 1900; Tyler, 1953), it has been suggested that inactive, senescent *Arbacia* sperm can be reactivated by treatments which would not be expected to replenish "energy reserves" (*e.g.* dilution by fresh sea water [Gray, 1928a] or suspension in sea water in which eggs had been incubated [Cohn, 1918; Hathaway, 1963]). However, no satisfactory explanations have been offered which explain the well-documented inactivation, or the less clearly defined reactivation of sperm. Thus, for example, an inhibitory effect of heavy metals has been invoked to explain the inactivation of sperm (Rothschild and Tyler, 1954). While metals certainly may inhibit motility,

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* *We dedicate this paper to the late H. Burr Steinbach, the mentor of two of us (PD & LN).*

Abbreviations: see Table I.

their removal is unlikely to be the basis for the increase in oxygen consumption upon dilution in fresh sea water, the "dilution effect" (Gray, 1928b). Possible clues come from studies in which a wide variety of agents have been shown either to increase the motility of freshly diluted *Arbacia* sperm or slow the onset of inactivation (*e.g.*, Branham, 1966; Steinbach, 1966; Tyler and Tyler, 1966; Nelson, 1972a, 1978; see also Steinbach and Dunham, 1961). Similar observations on motility have been made on sperm from various mammalian and avian species (Schindler and Nevo, 1962; Wales and White, 1962; Liess and Grove, 1963; VanDemark and Koyama, 1963; Garbers *et al.*, 1971; Bavister, 1981).

The normal inhibition of polyspermy in *Arbacia* eggs and those of many other species has long been recognized but remains poorly understood. Two general types of mechanisms might be involved: 1) the surface of the egg, or some portion of it, may be altered subsequent to binding or fusion of one sperm, thereby reducing the probability of penetration of additional sperm; 2) subsequent to contact with one sperm the egg may release an agent or agents which reduce the fertilizing capacity of neighboring sperm. Mechanisms of both types have been proposed. For example, F. R. Lillie described the reversible agglutination of *Arbacia* sperm by a substance released from eggs which he called fertilizin (Lillie, 1913, 1919). Lillie also appreciated that the egg's cortical reaction is too slow to be the only process at the surface of the egg operating to prevent polyspermy (see recent reviews containing discussions of polyspermy by Austin, 1978; Epel, 1978; Schuel, 1978; and Dale and Monroy, 1981).

Doubts have been expressed about the existence of a rapid block to polyspermy in sea urchin eggs (Hagström and Allen, 1956; Dale and Monroy, 1981). A block to polyspermy associated with electrical depolarization, first proposed by Gray in 1922, has recently been demonstrated in *Strongylocentrotus* eggs (Jaffe, 1976); the depolarization and associated block to polyspermy have been suggested to depend on Na (Schuel and Schuel, 1981). However, the validity of these conclusions has been called into question (Dale and Monroy, 1981).

It was shown nearly a century ago, and confirmed many times since, that a wide variety of chemical agents can induce polyspermy in sea urchin eggs (Hertwig and Hertwig, 1887; Just, 1928; Clark, 1936; Rothschild, 1954; Hagström, 1956; Schuel *et al.*, 1976; Coburn *et al.*, 1981). Polyspermy has now been observed in a wide range of animals (mammals as well as invertebrates). The nature of agents with such reactivity is so diverse as to support no single proposed mechanism for polyspermy; rather, the diversity suggests multiple mechanisms by which polyspermy is induced and therefore redundant mechanisms for the normal block to polyspermy. For example, Hertwig and Hertwig (1887) and Hagström and Allen (1956) induced polyspermy with nicotine and Clark (1936) and Hagström (1956) did so with strychnine; Nelson demonstrated stimulation of motility of freshly diluted sperm by nanomolar concentrations of nicotine (Nelson, 1978) and by micromolar concentrations of strychnine (Nelson, 1972a). However, the higher concentrations which may have been necessary for induction of polyspermy (*e.g.* Clark, 1936) inhibit motility (Nelson, 1972a, 1978; Jaffe, 1980).

An agent with very different reactivity, trypsin inhibitor from soy beans, has been shown to induce polyspermy (Hagström, 1956; Vacquier *et al.*, 1972; Schuel *et al.*, 1976). This agent might act by interfering with the cortical reaction (one of the initial events in fertilization), consistent with a role of an esteroprotease in this reaction (Grossman *et al.*, 1973). In another intriguing observation, polspermy was induced by catalase (Coburn *et al.*, 1981), suggesting that the block to polyspermy is due to release of $H_2O_2$ from the eggs during the fertilization reaction.

We have found that any of several proteins can reactivate inactive sperm. Reactivation was judged from measurements of fertilizing capacity and of motility. The same proteins also induced polyspermy over a similar range of concentrations. The diverse properties of the proteins (from the enzyme catalase to the relatively inert gelatin) suggest that their mode of action is nonspecific. Our results represent the first clear demonstration of reactivation of the fertilizing capacity of inactive sperm. We also provide evidence that induction of polyspermy and reactivation of sperm have a similar basis. However, it is probable that more than one mechanism exists for induction of polyspermy (and therefore that there is more than one mechanism for the physiological block to polyspermy). Finally, the nature of our effective agents requires a reexamination of mechanisms which have previously been proposed for the modulation of the activity of sperm.

## MATERIALS AND METHODS

*Gametes.* Spermatozoa and eggs were obtained from mature sea urchins (*Arbacia punctulata*) collected by the Department of Marine Resources of the Marine Biological Laboratory.

Sperm: Electrodes from a 12 v A.C. source were placed across the aboral surface of a male sea urchin for 30 sec or less. The sperm released were rinsed into sea water (~15 ml). Numbers of sperm per ml were determined by absorbance of light at 540 nm in a Spectronic 20 Colorimeter (Bausch and Lomb) (Nelson, 1972a).

Eggs: Female sea urchins were inverted over beakers of sea water (50 ml) and injected periviscerally with ~1 ml of 0.5 *M* KCl. The eggs released were washed twice in sea water by suspension and sedimentation at 1 × *g*. Numbers of eggs/ml were calculated from the packed volume of eggs after centrifugation to constant volume with a hand centrifuge and the mean diameter of *Arbacia* eggs (~75 μm; Harvey, 1956).

*Inactivation of sperm by aging.* Suspensions of sperm diluted in sea water to about $30 \times 10^6$ sperm/ml were allowed to stand for one to two days at room temperature (22–25°C).

*Fertilizing capacity of sperm.* As a measure of the function of sperm, fresh and inactivated, their capacity to fertilize eggs was measured. The method was similar to that of Lillie (1915). These assays were carried out in plastic Petri dishes (35 mm × 10 mm) at room temperature in a total, final volume of 2 ml. Appropriate volumes of sperm suspension (0.05–0.2 ml) were added to give $\sim 10^6$ sperm/ml, final density. Agents to be tested for their effect on fertilizing capacity were then added, and the mixtures were incubated at room temperature, usually for 6 minutes. Then eggs were added (0.1–0.2 ml of stock suspension) to a final density of 25,000 eggs/ml. After incubation for 5 minutes, fertilizing capacity of the sperm was assayed by counting the number of eggs (in a field of 100) with a raised fertilization membrane. (In some experiments the eggs were counted again after 90 min for 2-cell stages as a measure of "normal" fertilization.) Bright field illumination in a compound microscope was used at low power. During this study, 49,400 eggs in all were scored (*c.f.* Weissmann, 1981).

We observed that SBTI modified the cortical reaction which occurs upon fertilization, confirming the observations of others (see Epel, 1978, and Schuel, 1978). The lifting of the fertilization membrane was much less pronounced than in control eggs. However fertilization was not prevented by SBTI and subsequent divisions were not modified. None of the other proteins tested modified the cortical reaction.

*Motility of sperm.* This was determined by a method described earlier (Nelson, 1972b). Aged sperm were first incubated (~6 min) with agents to be tested for their effect on motility. Then the sperm suspension (at 4–8 × $10^6$/ml) was placed in a low centrifugal field (120 × *g*) at room temperature for 4 minutes. Under these conditions (in which formaldehyde-killed sperm do not sediment), motile sperm tend to move in a centrifugal direction and the immotile sperm remain in the supernatant suspension. Thus the optical density (at 540 nm in a Spectronic 20 Colorimeter) of the supernatant suspension (containing the immotile cells) is inversely related to motility (Nelson, 1972b).

*Polyspermy.* Polyspermy was assayed in plastic Petri dishes set up as described above for measurement of fertilizing capacity. Sperm were incubated (6 min) with agents to be tested for their promotion of polyspermy. Then eggs were added and the mixtures were incubated for 45–60 minutes. In all cases at least 90% of the eggs were fertilized, and at least 55% (and generally more than 80%) of the eggs cleaved, either reaching the normal two-cell stage, or being readily recognizable as an aberrant form typical of polyspermy (Just, 1928; Clark, 1936). Scoring was made of fields of 100 eggs for: a) unfertilized eggs; b) fertilized eggs, 1-cell stage; c) normally fertilized eggs, 2-cell stage; and d) polyspermic eggs.

*Proteins.* The proteins tested for their effects on spermatozoan function were added to the assay suspensions from stock solutions made in sea water (up to 10 mg/ml). Table I lists the proteins employed, their approximate molecular weights, and their commercial sources.

*Statistical tests.* The randomization test for matched pairs (two tailed) was used to determine levels of significance of difference (*P*) from controls caused by treatments with proteins. This is a nonparametric test with 100% power efficiency (Siegel, 1956). Standard errors of means (SEMs), not used in tests for significance of differences, are shown to indicate variability between experiments. The number of separate experiments (on different preparations of cells) is given by "n".

## RESULTS

*Reactivation by proteins of aged sperm: fertilizing capacity.* We confirmed that *Arbacia* sperm diluted in sea water and aged for a number of hours become inactive

TABLE I

*Proteins employed in studies on function of* Arbacia *spermatozoa, the approximate molecular weights of the proteins, and their commercial sources.*

| Protein | Molecular weight | Commercial source | Abbreviation |
|---|---|---|---|
| catalase (prepared from bovine liver) | 250,000 | Sigma Chemical Co., St.Louis, MO | CAT |
| crystalline bovine serum albumin | 60,000 | Sigma | BSA |
| Cohn fraction V (from bovine serum) | 60,000 | Sigma | CFV |
| superoxide dismutase | 32,000 | Miles Laboratories Ltd., Rep. of S.A. | SOD |
| Soy bean trypsin inhibitor (type I-S) | 21,000 | Sigma | SBTI |
| gelatin (granular) | (indeterminate) | Matheson Coleman & Bell, Norwood, OH | GEL |

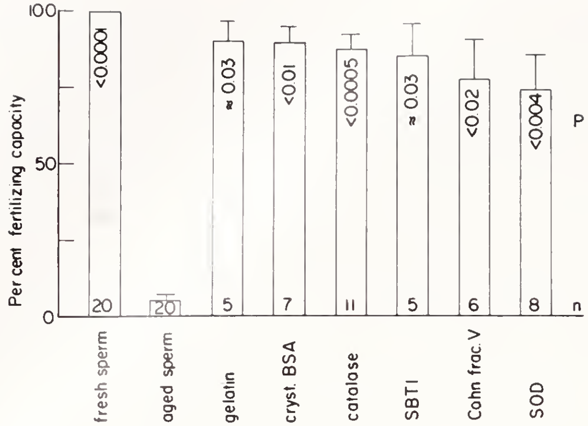

FIGURE 1. Reactivation of the fertilizing capacity of aged *Arbacia* sperm by various proteins, all at 0.5 mg/ml. Procedures for inactivation of sperm by aging and for determination of fertilizing capacity are given in Materials and Methods. Error bars indicate SEMs; n, numbers of experiments. *P* is the level of significance of difference from aged sperm not treated with protein (randomization test for matched pairs).

as judged by their fertilizing capacity. We then discovered that brief incubation of these aged sperm with any of several proteins dramatically restored their fertilizing capacity. Figure 1 shows measurements of fertilizing capacity of fresh sperm, aged sperm (one or two days), and aged sperm incubated 6 minutes with six different proteins (all at 0.5 mg/ml), both enzymatic (CAT and SOD) and nonenzymatic. In preliminary experiments, one other protein, ovalbumin, also reactivated aged sperm. No other proteins were tested.

Preliminary determinations of the time course of reactivation indicated that the full effect was achieved well before 6 minutes. Unfortunately the time required for fertilization by fully active sperm makes an accurate determination of the time course of reactivation impossible.

Figure 2 shows the effect on fertilizing capacity of aged sperm of the proteins in Figure 1 as a function of protein concentration (weight/volume). Reactivating activity was detectable at 5 $\mu$g/ml or less. The curves for the various proteins are similar with concentrations expressed as weight/volume despite the wide range of their molecular weights (21,000–250,000; see Table I).

*Reactivation of aged sperm: motility.* Figure 3 shows measurements of motility of aged sperm reactivated by brief incubation with each of the six proteins used to reactivate fertilizing capacity. The motility of the aged sperm was about 25% of the motility of freshly diluted sperm. All of the proteins increased the motility of aged sperm. Reactivation, judged by motility, is less dramatic in quantity than the reactivation of fertilizing capacity, but it is striking nevertheless.

Despite reactivation of aged, inactive sperm by proteins, we observed, in a preliminary experiment, that aging the sperm in the presence of the proteins did not protect them from eventual inactivation as judged by their motility.

*Induction of polyspermy by proteins.* Table II shows the results of three typical experiments on induction of polyspermy by three proteins. In addition to the results on polyspermy, Table II shows that the treatment with proteins did not affect fertilization or cleavage. That the variability in per cent polyspermy among these three

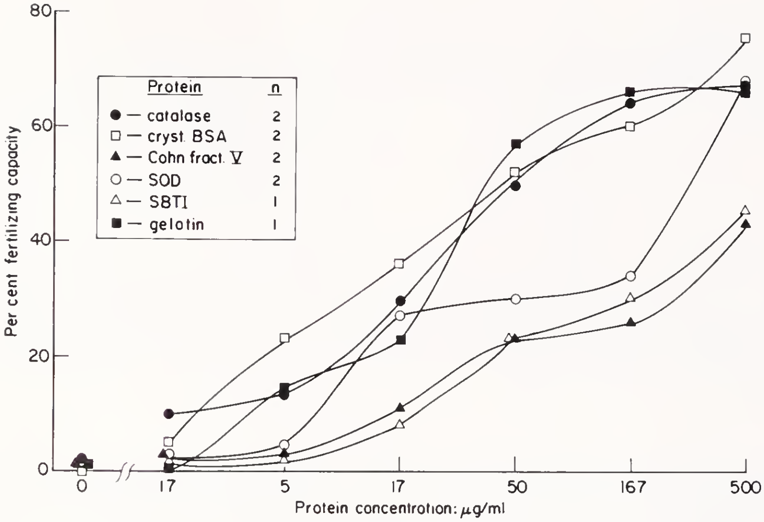

FIGURE 2. Reactivation by proteins of fertilizing capacity of aged *Arbacia* sperm as a function of protein concentration (μg/ml). Procedures for inactivation and for determination of fertilizing capacity are given in Materials and Methods. Protein concentrations on the abscissa are plotted in a logarithmic scale. The inset shows the symbols for the proteins and n, the numbers of experiments for each.

experiments was great is indicated by the standard errors. In these three experiments, however, in no instance was the level of polyspermy induced by a protein in a suspension of sperm and eggs less than 2-fold greater than its control.

Figure 4 shows levels of polyspermy induced by the six proteins (all at 2 mg/ml) in a series of experiments (not every protein was tested in each experiment). Again,

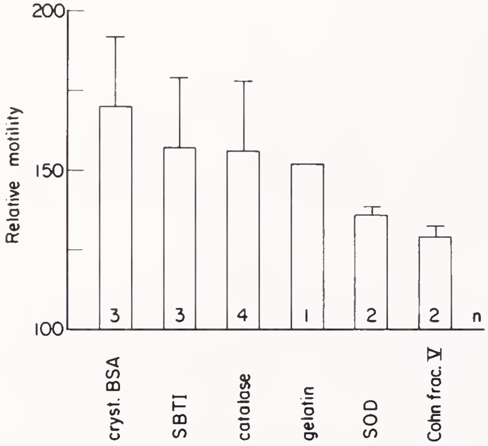

FIGURE 3. Motility of aged *Arbacia* sperm reactivated with proteins, presented relative to the motility of aged sperm, and determined as described in Materials and Methods. The motility of aged sperm not treated with proteins, set at 100, was approximately 25% of the motility of fresh sperm. Numbers of experiments are shown; the error bars show SEMs for BSA, SBTI, and CAT (n ≥ 3), and total ranges for SOD and CFV (n = 2).

TABLE II

*Induction of polyspermy by proteins in mixtures of* Arbacia *sperm and eggs.*

| Condition | Per cent fertilized eggs | Per cent cleavage among fertilized eggs | Per cent polyspermy among cleaved eggs |
|---|---|---|---|
| control | 99.0 ± 0.6 | 93.7 ± 3.8 | 2.3 ± 1.4 |
| catalase | 98.3 ± 1.7 | 92.3 ± 2.3 | 22.3 ± 9.4 |
| gelatin | 98.0 ± 2.0 | 93.3 ± 1.8 | 9.3 ± 2.0 |
| crystalline albumin | 99.3 ± 0.3 | 93.3 ± 2.0 | 17.1 ± 5.9 |

Shown are the per cent of eggs fertilized, the per cent of fertilized eggs which had undergone cleavage (either to the normal 2-cell stage or to aberrant polyspermic forms), and the per cent of cleaved eggs which were polyspermic. Results are from suspensions of sperm and eggs incubated with catalase, gelatin, or crystalline albumin (all at 2 mg/ml), and control suspensions. Values are means ± SEMs from 3 experiments.

in no instance was the per cent polyspermy induced by a particular protein less than 2-fold greater than its control (and generally they were much higher). In nine of the thirteen experiments, no polyspermy was observed among 100 eggs in the control suspensions; the highest control level was 4.7%. (The low control levels of polyspermy in Table II and Figure 4 show that there was no problem with overinsemination.) In the four experiments with SOD (the protein least effective at 2 mg/ml in inducing polyspermy), the highest control level was 1.2%, and three were zero; the lowest level with SOD was 2.1%, and its associated control was zero. As shown in Figure 4, there was a high probability of significance of the effects of all the proteins with the possible exception of SOD. In preliminary experiments ovalbumin also induced polyspermy (11.3%; control, 2.2%; n = 3).

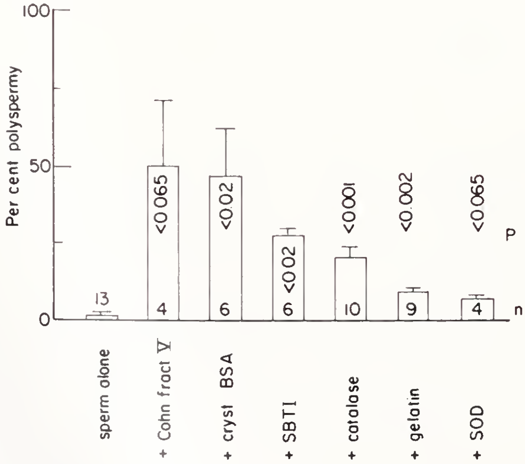

FIGURE 4. Induction of polyspermy in *Arbacia* eggs by various proteins, all at 2 mg/ml. Sperm were freshly diluted and had 100% fertilizing capacity. The experimental design for inducing and quantifying polyspermy is given in Materials and Methods and is illustrated in Table II. The values are means of the per cent polyspermic eggs (aberrant cleavage) of cleaved eggs 45–60 min after mixing sperm and eggs. Error bars represent SEMs; numbers of determinations are also shown. *P* is the level of significance of difference from the control, *i.e.* sperm alone (randomization test for matched pairs).

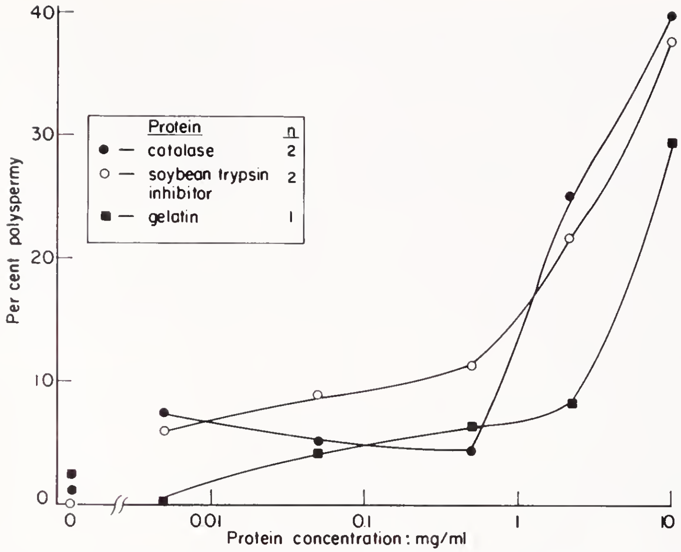

FIGURE 5. Induction by catalase, SBTI, or gelatin of polyspermy in *Arbacia* eggs as a function of the concentrations of the proteins (mg/ml). Sperm were freshly diluted, and had 100% fertilizing capacity. The experimental design is given in Materials and Methods and is illustrated in Table II. The ordinate, "per cent polyspermy," is the per cent of cleaved eggs which were polyspermic (aberrant cleavage). Protein concentrations on the abscissa are plotted in a logarithmic scale. The inset shows the symbols for the proteins and n, the numbers of experiments for each.

Figure 5 shows the dependence of induction of polyspermy on concentration for three of the proteins. The curves describing the dependence on concentration expressed as w/v are similar to one another despite a difference of an order of magnitude in molecular weight between catalase and SBTI (see Table I). The results confirm and extend the recent results from similar experiments (Schuel *et al.*, 1976; Coburn *et al.*, 1981; Schuel and Schuel, 1981), in which catalase and SBTI induced polyspermy. Figure 5 shows that these two proteins induced polyspermy at the lowest concentration tested (5 μg/ml), nearly as low as the concentration (1.7 μg/ml) at which most of the proteins could reactivate the fertilizing capacity of the aged sperm (Fig. 2). The results are in conflict with studies by Coburn *et al.* (1981) and Schuel and Schuel (1981), in which a failure of BSA to induce polyspermy was reported. However, Coburn *et al.*, (1981) reported that boiled catalase (concentration not given) induced <20% polyspermy (and therefore presumably some polyspermy).

## DISCUSSION

In this study we show that proteins can promote activity of *Arbacia* sperm. The activities measured were fertilizing capacity, motility, and polyspermy. The proteins stimulated fertilizing capacity and motility of inactive sperm, and induced polyspermic fertilization of eggs by fresh sperm.

Brief incubations (6 minutes or less) with any of six proteins reactivated aged sperm, and no proteins were tested which were ineffective. The diversity of the proteins makes clear the limited specificity of their effect: two are enzymes (CAT, SOD), one is an enzyme inhibitor (SBTI), two are nonenzymatic serum proteins (BSA, CFV), and one (GEL; boiled collagen) is particularly lacking in reactive groups (Miller and Gay, 1982).

The dose-response curves for activation of aged sperm (and for induction of polyspermy, though only 3 proteins were tested) show that the concentration dependence is not on a molar basis, but on concentration as weight/volume. For example, CAT and SOD were about equally effective in reactivating aged sperm at 17 μg/ml (Fig. 2), though their molecular weights differ by nearly an order of magnitude. Similarly CAT and SBTI had comparable activities in inducing polyspermy below 0.1 mg/ml (Fig. 5). Despite this evidence for limited specificity, in most instances the proteins were active in promoting both functions at 5 μg/ml or less. These various considerations make difficult the task of deducing the mechanism of action of the proteins on sperm function. Prevention of binding of inhibitors seems possible but not likely: gelatin is as active in its effects on sperm function as the other proteins, but lacks reactive groups (gelatin is totally lacking in cysteine residues; Miller and Gay, 1982).

Metal chelators can delay inactivation of sperm (Rothschild and Tyler, 1954; Tyler and Tyler, 1966). The relative affinity of such a chelator as ethylene diamine tetracetate (EDTA) for $Cu^{++}$ (the probable inhibitor of sperm in sea water; Rothschild and Tyler, 1954) is 8–10 orders of magnitude higher than for Ca and Mg, the prevalent divalent cations in sea water ($\log_{10} K_{eq}$ of EDTA for Ca: 10.6; for Mg: 8.8; for Cu: 18.7; Martell and Smith, 1974). Therefore, even though the concentrations of Ca and Mg in sea water are ~4 orders of magnitude higher than the concentration of Cu, EDTA would have a much greater effect on [Cu] than on [Ca], and could thereby influence sperm function.

However, it is unlikely that proteins have so pronounced an effect. Most natural amino acids (except cysteine) have about the same affinity for Cu as EDTA has for Mg, and proteins lacking cysteine have a much lower affinity for Cu than do amino acids (for five different pentapeptides the $\log_{10} K_{eq}$ for Cu was about 5.4; Martell and Smith, 1974), and as stated several times above, gelatin, lacking cysteine residues, was as active as the other proteins which contain reactive groups, in its effect on sperm function.

Furthermore, if the mechanism of reactivation of aged sperm by proteins is similar to their mechanism of inducing polyspermy, then chelation of heavy metals cannot be the sole process involved; a role of heavy metals in the block to polyspermy appears unlikely.

Interference with (or binding to) inhibitory substances released from eggs (*e.g.* fertilizin) might explain induction of polyspermy, but cannot explain the activation of aged sperm which have not been in contact with eggs or their products.

Two recent preliminary studies on the effects of bovine serum albumin on rodent sperm suggest a role for proteins in capacitation, *i.e.* preparation for the acrosome reaction (Bavister, 1981; Go and Wolf, 1981). This suggestion may or may not be of relevance to *Arbacia* sperm.

Whatever the mode of action of proteins in inducing polyspermy, our results militate against the suggestions of Coburn *et al.* (1981) and Schuel *et al.* (1976) of specific enzymatic or enzyme inhibitory effects based on induction of polyspermy by CAT and SBTI, since serum albumins and gelatin are also effective (Fig. 5).

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# AN ECHINODERM VITELLARIA WITH A BILATERAL LARVAL SKELETON: EVIDENCE FOR THE EVOLUTION OF OPHIUROID VITELLARIAE FROM OPHIOPLUTEI

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## ABSTRACT

*Ophionereis annulata* (Le Conte) possesses a barrel-shaped, yolky, non-feeding vitellaria larva with transverse ciliary bands. However, the larva develops vestiges of skeletal structures that are characteristically present in feeding ophiopluteus larvae but absent from vitellariae. Thus, it is evident that the vitellaria of *O. annulata* is a modified ophiopluteus. The presence of a pluteus-like skeleton in a vitellaria larva is suggestive that the evolution of ophiuroid larval types proceeds in a gradual fashion with a larval skeleton remaining after other ophiopluteus structures are lost. Ophiuroid vitellariae have apparently evolved from ophiopluteus larvae. These findings support Mortensen's (1921) proposal that the lecithotrophic vitellaria is a modified pluteus and contradict the hypothesis (Fell, 1945; Williams and Anderson, 1975) that vitellaria larvae are divergent and distinct from the feeding ophiopluteus larvae.

## INTRODUCTION

Johannes Müller and other prominent zoologists of the 19th century discovered that each class of living echinoderms (with the exception of the crinoids) has a feeding (planktotrophic) larva with a distinctive body plan: the holothuroid auricularia, the asteroid bipinnaria, the echinoid echinopluteus, and the ophiuroid ophiopluteus. Müller (1850) also described a yolky, non-feeding (lecithotrophic) larva of ophiuroids. Because of its shape he called it a vermiform (*wurmformige*) larva; this type of larva was later renamed a vitellaria (Fell, 1945).

Certain species in all living echinoderm classes have yolky, non-feeding, larvae that differ strikingly from the better known feeding larvae. The crinoids that have been reared produce yolky, barrel-shaped vitellariae (also called doliolariae) with four or five transverse bands of cilia. Holothuroids have similar vitellariae (doliolariae), either as the definitive larva or as a secondary larval stage. Ophiuroid vitellariae look very much like the barrel-shaped crinoid and holothuroid larvae and generally have four transverse ciliary bands.

Fell (1945) introduced the term "vitellaria" for the larvae which he considered a divergent series possessing yolky, barrel-shaped bodies and transverse bands of cilia. Williams and Anderson (1975) drew a further distinction between lecithotrophic larvae that retain vestiges of feeding larva structures and a separate group of vitellaria larvae which lack any vestiges of feeding larva structures (such as a single ciliary band, mouth, anus, larval arms, and larval skeleton). In their view, reduced larvae such as *Peronella leseuri* echinoplutei or *Amphiura chiajei* ophioplutei are manifestly unlike the vitellaria larvae of the echinoid *Heliocidaris erythrogramma* or the ophiuroid *Ophioderma brevispinum*. Prior to these works, however, Mortensen (1898, 1921) treated ophiuroid vitellaria larvae as a variety of

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ophiopluteus. A consideration of the vitellaria of *Ophionereis annulata* (Le Conte) (Fig. 1A), as discussed below, supports Mortensen's contention that the ophiuroid vitellaria is a modified ophiopluteus.

## MATERIALS AND METHODS

I collected *Ophionereis annulata* at a depth of 12 m near Taboguilla Island in the Bay of Panama (Central America) on 15 May 1975. Specimens were taken to the Galeta Marine Laboratory of the Smithsonian Tropical Research Institute on the Carribbean Coast of Panama. A female ophiuroid, held in the laboratory in a fingerbowl with sea water, spawned spontaneously two days after collection. Its oocytes were fertilized using a dilute suspension of spermatozoa from the dissected testes of a male specimen of *O. annulata.*

Temperature in the laboratory cultures was 24–26°C, approximately that of the water at the collecting site. The process of larval development in these cultures is described from sketches of live specimens, from fresh squash preparations examined under standard and polarized illumination, and from preserved samples.

## RESULTS

The ova of *Ophionereis annulata* are round, 0.24 mm in diameter, and pale yellow-green to yellow-brown. They were denser than sea water, settling to the bottom of the culture vessel. Within 1.5 h after fertilization the embryos reached the 8-cell stage, and blastulae 0.27 mm in diameter developed by 5 h. Each blastula almost filled the vitelline membrane and did not move within it.

Swimming larvae were found near the bottom of the culture vessels by 10 h after fertilization, and by 12 h some larvae swam near the surface of the water. A 24-h gastrula was about 0.31 mm long, 0.23 mm wide, and somewhat wedge shaped with a blastopore at the center of the broadened posterior end. It swam with the narrow end foremost, rotating clockwise around the long axis of the body.

Several important changes were noted 36 h after fertilization. The blastopore was no longer visible, a hydropore penetrated the mid-dorsal surface of the larva, and internally, a branching hydrocoel encircled the presumptive oral area.

A triradiate level skeleton had appeared in each posterior corner of the larval body by 34 h (Fig. 1B). In the 36-h larva, the pair of spicules had grown, and in specimens 38 h old the three branches of each elongate larval skeletal element presumably corresponded to the body, postoral, and posterolateral rods of the pluteus skeleton (Fig. 1C). The more complex branching pattern of the 48-h larval skeleton may be an indication of the formation of homologues of the posterodorsal, anterolateral, and transverse rods of the ophiopluteus skeleton (Fig. 1D).

A pentaradiate ophiuroid rudiment (*i.e.* the developing adult body), on the midventral surface of the 38-h larva, below the branched hydrocoel, possessed a concave central oral area and tube foot buds. The entire surface of the 38-h larva including the ophiuroid rudiment was ciliated. Thickened, more densely ciliated ridges were present at the posterior end of the larval body. Apical cilia longer than the body cilia projected from the anterior end of the larva.

By 48 h the hydropore was no longer visible. At that stage the larva was approximately 0.42 mm long and 0.30 mm wide. The larval skeleton was displaced from the posterolateral corners of the larva, possibly through the growth and torsion of the ophiuroid rudiment preceding metamorphosis. Elements of the adult ophiuroid skeleton were conspicuous in squash preparations. They had appeared as tri-

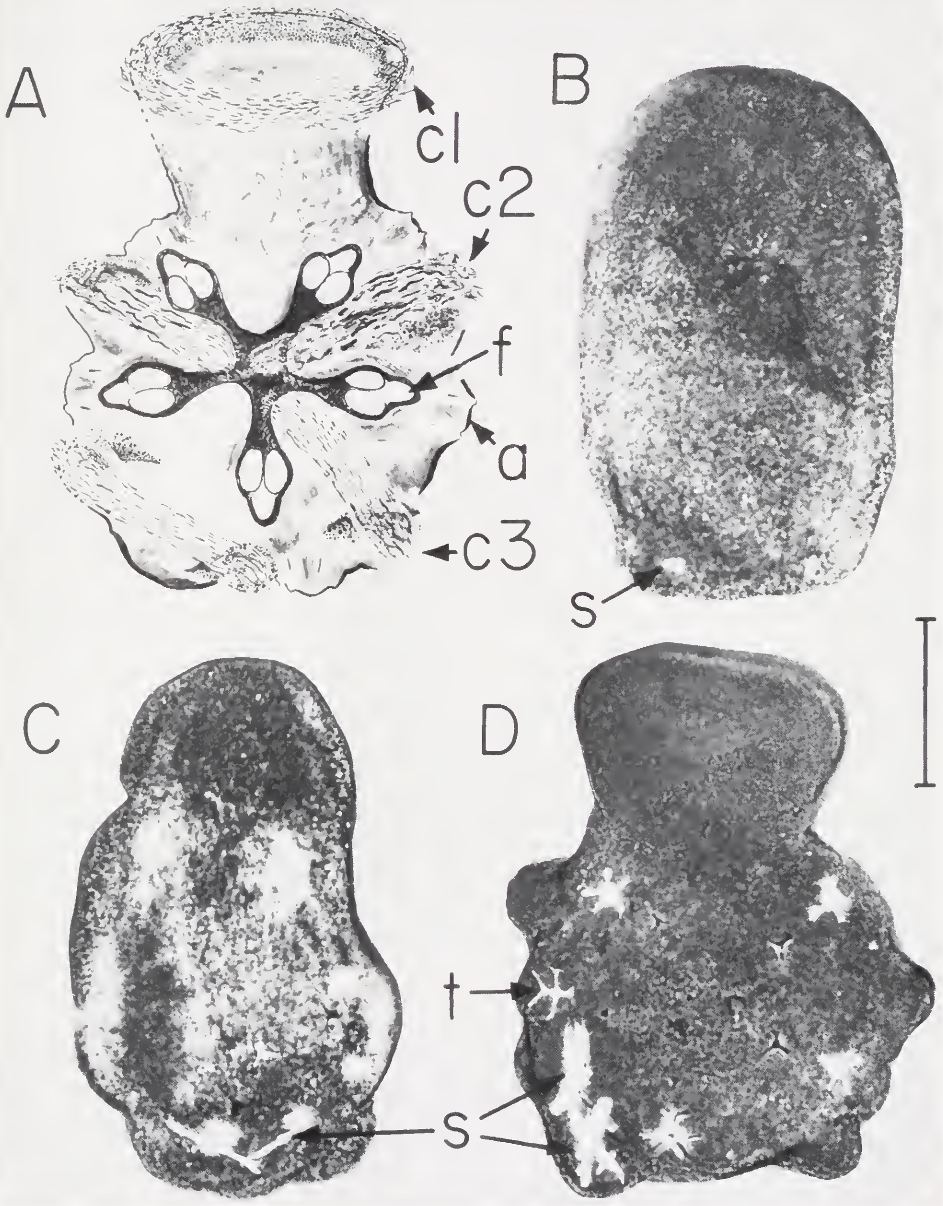

FIGURE 1. (A) 62-hour vitellaria larva of *Ophionereis annulata* drawn from life; $c_1$, anterior ciliated band on the funnel-shaped preoral lobe; $c_2$ and $c_3$, posterior ciliated bands on the ophiuroid rudiment; f, triad of the two buccal tube feet and a terminal tube foot lying between the epineural folds; a, arm tip of the ophiuroid rudiment containing a terminal arm plate. B–D are photographs of squash preparations of specimens viewed with polarized illumination that makes skeletal elements appear white and soft tissues of the larva appear dark: s, larval skeleton; t, terminal arm-plate of the definitive ophiuroid skeleton. (B) 34-hour larva with paired posterolateral rudiments of the larval skeleton. The hydrocoel is a scalloped structure at the center of the larva. (C) 38-hour larva with thin, slightly branched larval skeleton and five triradiate terminal arm-plates. Bulging projections at the posterior end of the larva will develop into ciliated bands. (D) 48-hour larva with branched larval skeleton and pentaradiate terminal arm-plates. The preoral lobe and ciliated ridges are seen in silhouette. Length of the scale is 0.10 mm.

radiate spicules by 38 h, and by 42 h they formed multibranched terminal arm plates as well as central, radial, and oral plates of the disc (Fig. 1C).

By 48 h the preoral lobe of the vitellaria formed a funnel shape, giving the larva a distinctive appearance (Fig. 1D). The larva had only three ciliary bands. The absence of a fourth small ciliary band on an elongation of the preoral lobe distinguishes *O. annulata* from other ophiuroid vitellariae, even from the vitellaria of *Ophionereis squamulosa* described by Mortensen (1921).

At 48 h cilia had disappeared from the ophiuroid rudiment. The remainder of the larval body was ciliated, but cilia were concentrated along the transverse ciliary bands. As the ophiuroid rudiment matured, cilia disappeared from most of the larval body, and by 62 h, ciliation was restricted to well-defined bands encircling the larva (Fig. 1A). Ciliary bands on the 72-h larva were opaque yellow-green, but other areas of the larval body, particularly the preoral lobe, had lost their yolky opacity.

The tube feet of 62-h larvae were papilliform, and by 72 h the three tube feet on each arm of the ophiuroid rudiment were capable of independent movement. Tube feet at the tip of the arm protruded from within the terminal arm plate and movements of the paired buccal tube feet set the jaw apparatus in motion.

The 88-h larva was about 0.42 mm long and 0.31 mm wide, approximately the same size as the 72-h larva. At this stage there was scanty ciliation on the ventral surface of the larvae, but lateral ciliation on the ciliary bands was still evident. The ophiuroid rudiment appeared opaque, due at least in part to the growing density of the adult skeleton and and the addition of new skeletal structures such as teeth. Larval skeletal elements, however, were completely resorbed by 88 h. They had reached their maximum size by 62 h, and over the next 26 h, the disappearing skeleton remained in one interradial sector of the ophiuroid rudiment.

The newly settled *Ophionereis annulata* moved by propelling the disc with the distal buccal pair of tube feet and the first pair of arm spines. The tips of the tube feet bear papillate extensions, much like the juveniles of *Amphioplus abditus* discussed by Hendler (1977). Within 24 h after settlement, portions of the larval body with yellow-green pigmentation were resorbed and the locomotory activity and agility of the juveniles increased. Within 8 days after settlement the stomach of the juvenile formed a distinct yellow structure within the disc. It is not known whether the pigmentation of the gut was from larval yolk or ingested food.

## DISCUSSION

Fully developed ophioplutei generally have four pairs of larval arms, and the abbreviated pluteus larvae of ophiuroids constitute a continuous reduction series with fewer arms than normal (Fell, 1945; Hendler, 1975). For example, *Amphiura filiformis* has three pairs, and both *Amphiura chiajei* and *Ophiothrix oerstedii* have only one pair of larval arms (Mortenson, 1921; Fell, 1945; Fenaux, 1963; Mladenov, 1979). Although the latter two species probably do not feed, they are clearly reduced ophioplutei that retain simplified pluteus arms, skeleton, and ciliation. There are species of brooding ophiuroids (*e.g.*, *Axiognathus squamatus* and *Ophionotus hexactis*) that have embryos with vestigial larval features, and other brooders have embryos lacking pluteus or vitellaria characteristics (*e.g.*, *Ophiomyxa brevirima*) (Mortensen, 1921; Fell, 1941, 1946). The brooded embryos with vestigial ophiopluteus structures have been considered to be modified ophioplutei (Fell, 1946).

Thus, there are reduced ophiuroid larvae which, like the reduced (and secondary) larvae of asteroids, holothuroids, and echinoids, retain certain salient vestiges of the feeding larva. These larval types would seem to bear no clear relation to the vitellaria larvae since vitellariae lack even vestigial feeding structures and are characterized by their barrel-like shape and multiple transverse ciliary bands that are used solely for locomotion, and not for feeding.

It is therefore surprising that Mortensen (1898) assigned ophiopluteus names to ophiuroid vitellariae, evidently believing that the yolky larvae were modified feeding larvae. Hamann (1901) objected to Mortensen's nomenclature, pointing to a lack of ophiopluteus structures such as larval skeleton in the vitellariae. Later, Mortensen (1921) detected irregular calcareous structures in the vitellariae of the ophiuroid *Ophiolepis cincta*, and he reiterated that the vitellaria was a reduced ophiopluteus. The larval skeleton of *Ophionereis annulata* by its structure and its position in the larva, is more like an ophiopluteus skeleton than are the spicules of *O. cincta* and, therefore, provides better evidence that vitellariae are derived from a feeding larval stage with a bilateral, branched larval skeleton. However, this deduction assumes that vitellaria and ophiopluteus larval skeletons are homologous.

Compelling evidence for homology of the vitellaria skeleton with the ophiopluteus skeleton lies in the fact that both are composed of branching, rod-like forms that develop at the posterolateral corners of the larval body and that are resorbed during metamorphosis. Moreover, the only echinoderm larvae with skeletal rods are vitellariae and plutei, and pluteus larval rods originate in a manner similar to the skeleton of *O. annulata*. It is highly unlikely that the form, location, and ontogenesis of larval skeletons would be duplicated by ophioplutei and vitellariae if the skeletal elements were not homologous.

Assuming that the skeletal structures of vitellariae and ophioplutei are homologous, is there additional evidence that the vitellaria is derived from the ophiopluteus? The necessary logical framework for a solution to this question was devised by Strathmann (1974, 1978) who argued that lecithotropic larvae are derived from feeding larvae. The most persuasive evidence for the evolution of lecithotrophic larvae from planktotrophic types is furnished by the fact that such a change requires a reduction rather than the repeated acquisition of extremely complex characters such as the single ciliary band feeding mechanism and a complete larval gut (Strathmann, 1974, 1978). Furthermore, some traits of planktrophic echinoderm larvae are unique to the phylum, whereas lecithotrophic larvae with transverse ciliary bands are simple forms that occur in unrelated taxa, indicating convergence (Jägersten, 1972; Strathmann, 1974).

The ophiopluteus skeleton, like the single ciliary band feeding mechanism, is a complex structure, presumably more readily lost than evolved. Thus, as suggested by the trend of progressive reduction in size and complexity of the larval skeleton shown in ophioplutei with 6, 4, and 2 larval arms, it might be expected that more highly simplified and modified ophioplutei (*e.g.* vitellariae) could retain some vestige of an ophiopluteus larval skeleton.

As already mentioned, the same reasoning has been applied in treating the relationship between ophioplutei and the specialized embryos of brooding species such as *Axiognathus squamatus* and *Ophionotus hexactis*. The paired skeletal elements in these embryos are regarded as vestiges of an ophiopluteus skeketon. Moreover, the formation of coelomic cavities in the ophiuroid vitellariae also appears to be a simplification of the process of coelomogenesis in the ophioplutei (Grave, 1916). Therefore, I regard the larval skeleton in the vitellaria of *Ophionereis annulata* as a vestigial rather than as a neomorphic (*i.e.*, newly evolved) structure.

The vitellaria of *Ophionereis annulata* provides the best indication that the ophiuroid vitellaria evolved from an ophiopluteus form. The continuity of the planktotrophic, reduced, and vitellaria larval forms evidenced by the skeleton of the *O. annulata* vitellaria negates the distinction drawn by Fell (1945) and Williams and Anderson (1975) between vitellariae and feeding larvae, and I consider these various ophiuroid larval forms to be homologous.

The presence of a vestigial larval skeleton in *Ophionereis annulata* implies that the loss of feeding larval structures during the evolution of yolky larvae may be a gradual process. Most vitellaria larvae have lost the larval digestive tract, arms, and single ciliary band, as well as the larval skeleton of the ancestral ophiopluteus form. However, the retention of a larval skeleton in *Ophionereis annulata* (and perhaps in *Ophiolepis cincta*) suggests that the larval skeleton may be lost later than other larval structures. In the vitellaria of the related species *Ophionereis squamulosa*, loss of pluteus larval structures is complete. Mortensen (1921) did not mention a larval skeleton in *O. squamulosa* and I have reared *O. squamulosa* (unpub. obs.) and found no trace of the skeleton. Therefore, the larva of *O. squamulosa* is presumably a more advanced form than the larva of *O. annulata*.

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# *IN VITRO* STUDIES ON THE EFFECTS OF CELL-FREE COELOMIC FLUID, CALCIUM, AND/OR MAGNESIUM ON CLUMPING OF COELOMOCYTES OF THE SEA STAR *ASTERIAS FORBESI* (ECHINODERMATA: ASTEROIDEA)

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## ABSTRACT

In asteroid echinoderms the loss of coelomic fluid due to injury is prevented by the clumping of coelomocytes at the site of the wound. Plasma (cell-free coelomic fluid = CF) coagulation has not yet been demonstrated in these animals. An *in vitro* system was used to quantify the effects of CF, $Ca^{2+}$, and/or $Mg^{2+}$ on coelomocyte clumping in the sea star *Asterias forbesi.*

The results show that the coelomocytes of *A. forbesi* require threshold levels of $Ca^{2+}$ and/or $Mg^{2+}$ for clumping *in vitro*, and these levels depend on whether the ions are used separately, in combination, or as components of CF. The findings also suggest that the *in vitro* coelomocyte clumping is mediated by a factor present in CF which requires $Ca^{2+}$ and $Mg^{2+}$ to be effective. A two-phase clumping, consisting of a fast phase followed by a slow phase, is also demonstrated.

The observed biphasic clumping is explained by the existence of two functional subpopulations among the coelomocytes which differ in their permeability characteristics and ability to establish surface adhesiveness for clumping. Morphological identities of these two subpopulations remain to be ascertained.

## INTRODUCTION

While it is known that the clumping of the coelomocytes occurs as a means of hemostasis in asteroid echinoderms (sea stars) (see reviews by Endean, 1966; Needham, 1970; Belamarich, 1976; Kanungo, 1982), controversy exists as to the type of coelomocyte and the mechanism involved in such cellular clumping. (In this paper the terms "aggregation" and "agglutination" are used interchangeably with "clumping" of coelomocytes *in vivo* or *in vitro*, and the term "cell" is used to refer to the "coelomocyte.")

Boolootian and Giese (1958, 1959) maintained that bladder amoebocytes transformed into filiform amoebocytes which then agglutinated to form plasmodial clots in eight species of sea stars which they investigated. The filiform stage was, therefore, viewed as a precoagulant phase. Johnson and Beeson (1966) on the other hand, reported that the filiform stage was not required to initiate or to maintain coelomocyte clumps in the sea star *Patiria miniata*.

In analyzing the mechanism of of coelomocyte agglutination, Boolootian and Giese (1959) also observed that the agglutination was not dependent on calcium but on the formation of disulfide linkages. However, Jangoux and Vanden Bossche

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Abbreviations: CF, cell-free coelomic fluid (plasma); CMFSS, calcium- and magnesium-free salt solution; Hepes, N-2-hydroxyethylpiperazine N-2-ethanesulfonic acid; NEM, N-ethylmaleimide.

(1975) reported that certain amounts of calcium were required to induce coelomocyte clumping in *Asterias rubens.*

Factors other than calcium have also been implicated in sea star coelomocyte clumping *in vitro* (Boolootian and Giese, 1959; Jangoux and Vanden Bossche, 1975; Kanungo, 1982) and *in vivo* (Bang and Lemma, 1962; Bang, 1970; Reinisch and Bang, 1971; Reinische, 1974). These studies suggest that a factor released at the time the animal is wounded or challenged with foreign materials mediates coelomocyte clumping. A factor capable of inducing clumping in the coelomocytes of *A. forbesi* has been isolated from the coelomocytes of this sea star (Prendergast and Suzuki, 1970; Prendergast *et al.*, 1974). However, the existence of a clotting factor in the plasma (coelomic fluid free of coelomocytes = CF) of sea stars has not yet been demonstrated. The present study provides some experimental evidence for the existence of such a factor in the CF and examines the role of calcium and magnesium in agglutination of the coelomocytes of *A. forbesi.*

## MATERIALS AND METHODS

### *Animals*

*Asterias forbesi* were purchased from the Marine Biological Laboratory, Woods Hole, Massachusetts. They were held in the laboratory at 12°C in 30-gallon aquaria with filtered, recirculating, continuously aerated sea water (salinity 30‰). No more than nine animals were kept in one aquarium, and the animals (wet weight 155–210 g) were used within ten days of their arrival in the laboratory.

Before the experiment the animals were screened under a low power dissecting microscope for surface wounds, and those without any visible wounds or abnormalities were used.

### *Collection of coelomic fluid*

Two or three sea stars were removed from the holding tank, placed in a pail containing fresh sea water at room temperature (22°C), and held there for about 0.5 h.

The animal was blotted with a soft sponge and weighed. It was then held upright to allow the coelomic fluid to accumulate in the downward-hanging arms. When the arms were visibly swollen, the dermal papullae near the tip of the swollen arms were abraded with a razor blade. Coelomic fluid (1 ml) was allowed to drop into a 15-ml graduated centrifuge tube, coated inside with a thin layer of paraffin (melting point 60°C), which held 9 ml of calcium- and magnesium-free salt solution (CMFSS) containing 15 m*M* ethylenediamine tetracetic acid (EDTA). CMFSS was prepared by dissolving the following components in a liter of glass-distilled water: NaCl, 25.5 g; KCl, 0.8 g; $Na_2SO_4$, 3.0 g; glucose, 3.0 g; and Hepes (N-2-hydroxy-ethylpiperazine N-2-ethanesulfonic acid), 2.86 g. The pH of CMFSS and CMFSS-EDTA solutions was adjusted to 7.4 with NaOH. The solutions were filtered through presterilized 0.22-$\mu$m Millipore filters and stored in sterile containers until use.

### *Preparation of coelomocyte suspension*

Soon after collection the coelomic fluid-CMFSS-EDTA solution was mixed by gentle pipetting several times through a Pasteur pipette. The resulting cell suspension was then centrifuged at $200 \times g$ for ten minutes. It was determined by light microscopy that the coelomocytes thus treated did not lyse or suffer visible damage.

Almost all cells in the suspension were separate and nonclumped. The supernatant was discarded and the cell pellet was resuspended in fresh CMFSS. Cell counts were made using a hemocytometer. Only nonclumped cells were counted, and the cell density was adjusted to about $10^6$ cells/ml. Usually coelomocytes from several animals were pooled to run replicate experiments.

### *The test system*

The experimental system we used to assess the sensitivity of coelomocytes to CF and to $Ca^{2+}$ and/or $Mg^{2+}$ is as follows. Cell suspension in CMFSS (10 ml) was placed in a 25-ml Erlenmeyer flask, the inside of which was coated with a thin layer of paraffin. This reaction flask was then placed in a shaker water bath at 20°C and agitated at 50–60 revolutions per minute. A count of nonclumped cells was made immediately after the cell suspension was placed in the flask. This count, taken at time zero, is referred to as the initial count. A test substance(s) (CF, $CaCl_2$, and/or $MgCl_2$) was then added (at various concentrations) to the suspension. Stock solutions (for CF see below) of the test salts were prepared in deionized water. Reagent grade chemicals were used in all experiments. The volume of a test substance(s) added to the reaction flask did not exceed 1% of the volume of cell suspension in the flask. The concentration of CF in the suspension is expressed as $\mu$l CF/ml CMFSS, whereas those of $CaCl_2$ and/or $MgCl_2$ are expressed in millimolar (m*M*) units of $Ca^{2+}$ and $Mg^{2+}$ assuming 100% dissociation of the salts in the test system. Control systems were prepared and incubated in exactly the same manner as the experimentals but contained appropriate volumes of deionized water in place of a test solution.

Nonclumped-cell counts were made at five-minute intervals for a total experimental period of 30 min. A significant decrease in nonclumped-cell number during an experiment was considered to be due to clumping of cells since other factors that could cause such a decrease in our *in vitro* system were eliminated (see below). Thus a concentration of a substance in the test system producing a significant decrease in nonclumped-cell count during the period of the experiment is referred to as "clumping concentration," and one that did not produce such a decrease is termed a "nonclumping concentration." Microscopic observations on samples taken from the reaction flasks were conducted along with the cell counts to determine if clumping of coelomocytes had actually occurred.

### *Tests for cell attachment and/or lysis*

Since coelomocyte attachment to the vessel wall and/or lysis of these cells could cause a reduction in nonclumped-cell counts in our *in vitro* system, the following experiments were performed to determine if these possibilities existed in our test system.

(a.) A portion of cell suspension containing a clumping concentration of CF (10 $\mu$l/ml) or of $Ca^{2+}$ and $Mg^{2+}$ (0.23 and 0.12 m*M*, respectively) was placed on paraffinized slides and incubated in a humidified chamber at room temperature for 30 min. The suspension was drained off and the slide was inspected under a compound microscope for possible cell attachment. (b.) The cell suspension containing the above mentioned clumping concentration of CF or $Ca^{2+} + Mg^{2+}$ was incubated for 30 min in a manner similar to other experimental systems described under the test system. After the incubation the cell suspension was centrifuged, and the pellet was resuspended in CMFSS containing 10 m*M* N-ethylmaleimide (NEM). A nonclumped-cell count was made to determine if the initial nonclumped-cell number was restored.

### *Preparation and assay of normal, dialyzed, and heated CF*

*Normal.* Coelomic fluid was collected in a precooled, paraffinized centrifuge tube by abrading the animals as described above. It was then centrifuged at 200 × *g* for 10 min. The cell pellet was discarded, and the supernatant was filtered through a sterilized 0.22-$\mu$m millipore filter and stored at −20°C in sterile containers.

Experiments were performed with different concentrations of CF ranging from 5 to 10 $\mu$l/ml (at graded concentration intervals of CF) to establish a cutoff point between nonclumping and clumping concentrations of CF.

*Dialyzed.* Fifty ml of normal CF was dialyzed against 500 ml of CMFSS for 48 h at 4°C with constant stirring. CMFSS was changed five times during the 48-h period. At the end of this period, CF was sterilized by filtration and stored as discussed above.

The clumping effectiveness of dialyzed CF was tested by adding the CF at 10 $\mu$l/ml to the test system. This concentration was chosen because experiments with normal CF suggested this was a clumping concentration.

*Heated.* Normal CF was heated for 15 min at 100°C then cooled, sterilized by filtration, and stored as described above. This CF was also assayed at 10 $\mu$l/ml for its clumping effectiveness.

### *Experiments with $Ca^{2+}$ and/or $Mg^{2+}$*

Calcium and magnesium were assayed in absence of CF by adding various concentrations of these ions, independently of each other, to the test system. From these experiments nonclumping and clumping concentrations for $Ca^{2+}$ and $Mg^{2+}$ were established. Similarly, nonclumping and clumping concentrations of $Ca^{2+}$ + $Mg^{2+}$ were determined by assaying the two ions in combination in the test system.

### *"Reconstitution" experiments*

These experiments were designed to test the clumping ability of dialyzed CF in the presence of $Ca^{2+}$ and $Mg^{2+}$. Two series of experiments, were performed. (i.) initially dialyzed CF at 10 $\mu$l/ml (a clumping concentration for normal CF) was added to the test system. The system was then incubated for 30 min after which $Ca^{2+}$ and $Mg^{2+}$ were added to yield concentrations of 0.1 m*M* and 0.6 m*M*, respectively. These specific ion concentrations were used because normal CF when added to the test system at a concentration of 10 $\mu$l/ml yields 0.1 m*M* $Ca^{2+}$ and 0.6 m*M* $Mg^{2+}$ (see below for determination $Ca^{2+}$ and $Mg^{2+}$ in CF). Subsequent to the addition of the divalent cations in the entire system was further incubated for another 30-min period. Nonclumped-cell counts were made at 5- or 10-min intervals from the beginning of the experiment to the end of the second 30-min incubation period. (ii.) The sequence of addition of dialyzed CF and $Ca^{2+}$ + $Mg^{2+}$ to the test system was reversed. First $Ca^{2+}$ and $Mg^{2+}$ were added to obtain concentrations of 0.1 m*M* and 0.6 m*M*, respectively. The system was then incubated for 30 min following which dialyzed CF at a concentration of 10 $\mu$l/ml was added. Subsequently, the entire system was further incubated for another 30-min period. Cell counts were made during the entire 60-min incubation period as described in (i). In these two series of experiments the initial time of reconstitution refers to the time when the final ingredient(s) (dialyzed CF or the divalent cations) was (were) added to the test system.

### *Coelomocyte viability tests*

After the experiment, viability of coelomocytes was determined by two separate methods: Trypan Blue exclusion method (Phillips, 1973), and visual observation of coelomocyte attachment and spreading on a glass surface. For the latter test a sample of postexperimental cells was placed on a clean glass slide and observed under a phase contrast microscope. Since only live coelomocytes can attach to a surface and spread by extending petaloid pseudopodia (formed by bladder coelomocytes which constitute over 90% of the total coelomocyte population [Kanungo, 1982]), those that attached and spread their pseudopodia were considered viable.

### *$Ca^{2+}$, $Mg^{2+}$, and osmolality determinations*

Normal and dialyzed CF were analyzed on a flame photometer (Coleman Model 51, Coleman Instruments, Perkin-Elmer Corp., Maywood, IL) for their $Ca^{2+}$ contents while their $Mg^{2+}$ contents were determined using an atomic absorption spectrophotometer (Perkin-Elmer Model 560, Perkin-Elmer Corp., Maywood, IL.)

The osmolalities of various solutions were determined using an Advanced Digimatic Osmometer (Advanced Instruments, Inc., Needham Heights, MA). The instrument operates on freezing point depression principle and gives a readout in milliosmoles/kg.

### *Statistical analysis and calculations*

Paired *t* tests were applied to compare cell counts at each time interval with that at zero time. Data were considered significant at the 95% confidence level.

In case where the number of nonclumped cells is expressed as % of the initial number, linear regression was used to determine the best fit lines (except where indicated otherwise).

The percent clumping was calculated by subtracting the mean cell count at a given time point from that at time zero (or at time 30 min in reconstitution experiments) and taking this difference as percent of the corresponding initial count.

## RESULTS

The assumption that a decrease in the number of single coelomocytes in our *in vitro* system was not due to attachement and/or lysis of cells but due to clumping must be justified, for if this assumption is not valid the results obtained by using this system would be meaningless.

The osmolalities of CMFSS and CF were 933 and 955 mOsmol/kg, respectively. They were, therefore, considered here as isoosmotic for all practical purposes. Thus, the coelomocytes could not have been osmotically stressed even though the coelomic fluid was diluted 10× with CMFSS during collection. Almost all cells remained viable during the experimental period as judged from post-experimental cell viability tests.

### *Cell attachment and/or lysis*

Microscopic observations showed that the coelomocytes did not attach to paraffinized slides. However, the same cells, when placed on clean glass slides, adhered to the glass surface, extended petaloid pseudopodia, and subsequently underwent transformation from bladder to filiform type.

TABLE I

*Changes in the number of nonclumped coelomocytes of the sea star* Asterias forbesi *in the test system (cell suspension in CMFSS) and after exposure to clumping concentrations of CF or* $Ca^{2+}$ + $Mg^{2+}$ *for 30 min* in vitro *followed by exposure to 10* mM *NEM.*

| Time (min) | Number ($\bar{x} \pm SD \times 10^{-4}$/ml) of nonclumped coelomocytes | | |
|---|---|---|---|
| | CMFSS | CF (10 μl/ml) | 0.23 m*M* $Ca^{2+}$ + 0.12 m*M* $Mg^{2+}$ |
| | *n*[+] = 9 | *n* = 6 | *n* = 6 |
| 0 | 104.00 ± 11.52 | 115.00 ± 8.17 | 125.83 ± 20.45 |
| 5 | 103.78 ± 18.53 | — | — |
| 10 | 101.00 ± 23.36 | — | — |
| 15 | 107.00 ± 22.46 | — | — |
| 20 | 100.89 ± 15.79 | — | — |
| 25 | 108.11 ± 21.38 | — | — |
| 30 | 104.56 ± 18.01 | 70.83 ± 8.86* | 70.83 ± 10.57* |
| | | ↓ + NEM | ↓ + NEM |
| 45 | — | 109.17 ± 4.49 | 123.33 ± 32.30 |

* Significant at 95% confidence interval in paired comparison *t* tests between the indicated mean ($\bar{x}$) and that at time zero.

[+] n = number of experiments.

There was also no significant decrease in the number of nonclumped cells during the 30-min experimental period when the cells were suspended in CMFSS (Table I) or in diluted CMFSS as in control flasks. However, the possibility existed that a test substance in clumping concentration in the system might cause attachment of the coelomocytes to the vessel (cell lysis does not occur in CF or in $Ca^{2+}$ and $Mg^{2+}$) and thus produce a decrease in nonclumped-cell count. This is discounted by the results given in Table I which show that after induction of clumping the initial number could be restored if the total cell population in the system was resuspended in NEM. Thus, the contention that the observed decrease in the number of coelomocytes in our test system was due to clumping and not due to attachment and/or lysis is fully justified.

## *Effects of CF*

*Normal CF.* CF of *A. forbesi* contains on the average 10 m*M* $Ca^{2+}$ and 60 m*M* $Mg^{2+}$. In our test system the number of nonclumped coelomocytes did not decrease significantly from that of the initial number when the cells were suspended in CMFSS containing CF at concentrations equal to or lower than 7 μl/ml (Table II). Increasing the CF concentration to 7.5 μl/ml or higher, however, resulted in significant decrease in nonclumped-cell number (Table II). The clumps produced by CF ranged from 2-cell to 5-cell aggregates.

The overall reduction in cell counts produced by CF at concentrations of 7.5 μl/ml and 10 μl/ml in a 30-min period were 22% and 33%, respectively (Fig. 1). Both reductions were significantly different from the corresponding counts at time zero (Table II). The greatest reduction in nonclumped cell counts was produced during the first five-min period when, on the average, a 19% clumping was observed. However, in the next 25-min period the two CF concentrations produced different patterns of clumping. Although a 3% increase was observed with the CF concen-

TABLE II

*Effects of various concentrations and treatment of CF on the number of nonclumped coelomocytes of the sea star* Asterias forbesi *at different time intervals under* in vitro *conditions.*

| | Number ($\bar{x}$ ± SD × $10^{-4}$/ml) of nonclumped coelomocytes | | | | | |
|---|---|---|---|---|---|---|
| | Concentration of CF (µl/ml) in test flasks | | | | | |
| Time (min) | 5 (n = 5) | 7 (n = 5) | 7.5 (n = 7) | 10 (n = 9) | 10 (Dialysed) (n = 8) | 10 (Heated) (n = 8) |
| 0 | 104.00 ± 10.18 | 102.20 ± 12.06 | 102.43 ± 6.70 | 112.11 ± 9.22 | 118.13 ± 16.76 | 119.00 ± 22.96 |
| 5 | 107.20 ± 10.00 | 95.80 ± 13.36 | 84.43 ± 7.73* | 88.11 ± 8.56* | 119.38 ± 15.30 | 117.83 ± 23.44 |
| 10 | 101.40 ± 10.95 | 92.20 ± 20.54 | — | 89.89 ± 17.34* | 113.75 ± 9.27 | 121.38 ± 40.73 |
| 15 | — | 86.20 ± 16.44 | 81.43 ± 7.73* | 84.22 ± 14.33* | 111.88 ± 18.86 | 117.00 ± 34.45 |
| 20 | 97.20 ± 16.36 | 91.00 ± 21.95 | 83.43 ± 8.52* | 82.33 ± 18.22* | 117.50 ± 19.69 | 110.50 ± 29.83 |
| 25 | 97.20 ± 12.25 | 88.60 ± 21.21 | 82.86 ± 7.38* | 80.00 ± 14.51* | 107.50 ± 19.36 | 107.13 ± 25.79 |
| 30 | 97.40 ± 8.16 | 97.80 ± 23.10 | 78.14 ± 10.25* | 72.67 ± 9.52* | 113.13 ± 10.88 | 111.25 ± 28.34 |

* Significant at 95% confidence interval.

tration of 7.5 µl/ml during this 25-min period, this was not significantly different from the 19% clumping produced in the initial period. (The regression line through these time points is, therefore, horizontal in Fig. 1.) On the other hand, a significant increase of 14% over the initial 19% was observed with a CF concentration of 10 µl/ml during the same 25-min period.

*Dialyzed and heated CF.* The osmolality of dialyzed CF was 933 mOsmol/kg, and such CF did not contain any detectable amount of $Ca^{2+}$ or $Mg^{2+}$. The number of nonclumped coelomocytes did not decrease significantly with the addition of 10

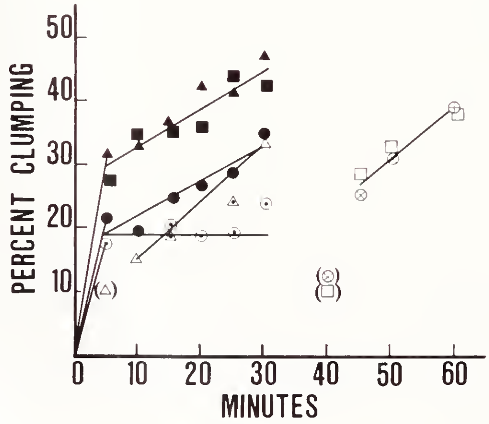

FIGURE 1. Effects of cell-free coelomic fluid (CF), $Ca^{2+}$, and/or $Mg^{2+}$ on clumping of coelomocytes of *Asterias forbesi in vitro.* The number of replicate experiments (n) performed in each category is shown in Tables II and III except those of the "reconstitution" experiments. ⊙ and ● for CF concentrations of 7.5 µl/ml and 10 µl/ml, respectively. ▲ and ■ for 0.45 m*M* $Ca^{2+}$ and 0.23 m*M* $Ca^{2+}$ + 0.12 m*M* $Mg^{2+}$, respectively. △ for 0.75 m*M* $Mg^{2+}$. Reconstitution experiments (see text for details): □, initial addition of dialyzed CF (n = 10); ⊗, initial addition of $Ca^{2+}$ + $Mg^{2+}$ (n = 8). Symbols in ( ) represent the corresponding mean cell counts that are not significantly different from those at time zero, or, in "reconstitution" experiments, from those at 30 min.

μl/ml of dialyzed or heated CF to the system, even though this concentration was well above the minimal clumping concentration of normal CF (7.5 μl/ml) (Table II).

### *Effects of $Ca^{2+}$ and/or $Mg^{2+}$*

There was no significant decrease in nonclumped-cell numbers with addition of 0.23 m*M* $Ca^{2+}$ or 0.50 m*M* $Mg^{2+}$ to the test system (Table III). Similarly, no reduction in cell number was observed when 0.1 m*M* $Ca^{2+}$ + 0.6 m*M* $Mg^{2+}$ were added to the system. However, addition of 0.23 m*M* $Ca^{2+}$ + 0.12 m*M* $Mg^{2+}$, or 0.45 m*M* $Ca^{2+}$ produced a reduction of 45% in a 30-min period, whereas 0.75 m*M* $Mg^{2+}$ reduced the cell number by about 32% from the initial during the same period (Fig. 1). In addition, the greatest amount of reduction was achieved during the first 5-min period when on the average a 30% decrease in nonclumped-cell counts was effected with the above concentrations of divalent cations (except with 0.75 m*M* $Mg^{2+}$). During the next 25-min period a reduction of about 15% in nonclumped-cell number was observed with 0.23 m*M* $Ca^{2+}$ + 0.12 m*M* $Mg^{2+}$ and with 0.45 m*M* $Ca^{2+}$. In Figure 1, one line is drawn through this 25-min period's time points because the calculated regression lines through the data points for the clumping concentrations of $Ca^{2+}$ + $Mg^{2+}$ and $Ca^{2+}$ are very close to each other. It is therefore reasonable to conclude that coelomocyte clumping requires higher concentrations of $Ca^{2+}$ or $Mg^{2+}$ when these ions are used individually than when they are used together and that the divalent cations act synergistically in regard to clumping.

With 0.75 m*M* $Mg^{2+}$ no significant decrease in nonclumped-cell count occurred during the initial 5-min period (Fig. 1). The large standard deviation associated with the mean suggests that there were excessive variations among the replicate counts taken at the end of the initial 5-min period (Table III).

### *Coelomocyte clumping pattern with "reconstituted" CF*

The reconstitution experiments showed a decline in nonclumped cells by about 40% in 30 min after reconstitution. In Figure 1 the line through these time points is drawn by estimation since the regression lines through respective data points with

TABLE III

*Effects of $Ca^{2+}$, $Mg^{2+}$, and $Ca^{2+}$ + $Mg^{2+}$ on the number of nonclumped coelomocytes of the sea star* Asterias forbesi *at different time intervals under* in vitro *conditions.*

| | Number ($\bar{x} \pm SD \times 10^{-4}$/ml) of nonclumped coelomocytes | | | | | |
|---|---|---|---|---|---|---|
| | Conc. (m*M*) of $Ca^{2+}$ | | Conc. (m*M*) of $Mg^{2+}$ | | Conc. (m*M*) of $Ca^{2+}$ + $Mg^{2+}$ | |
| Time (min) | 0.23 (*n* = 6) | 0.45 (*n* = 6) | 0.50 (*n* = 10) | 0.75 (*n* = 5) | 0.10 + 0.60 (*n* = 8) | 0.23 + 0.12 (*n* = 11) |
| 0 | 107.67 ± 21.57 | 113.29 ± 9.66 | 99.10 ± 10.79 | 112.20 ± 5.77 | 118.13 ± 14.35 | 122.73 ± 17.21 |
| 5 | 99.67 ± 25.73 | 78.43 ± 11.76* | 96.30 ± 24.15 | 101.00 ± 22.00 | 113.75 ± 13.64 | 88.91 ± 10.89* |
| 10 | 94.33 ± 21.88 | 76.43 ± 10.73* | 88.80 ± 22.18 | 95.40 ± 11.52* | 120.00 ± 9.35 | 80.00 ± 11.26* |
| 15 | 104.67 ± 25.77 | 72.71 ± 12.59* | — | 89.80 ± 13.86* | 113.75 ± 15.56 | 79.82 ± 9.29* |
| 20 | 104.33 ± 22.89 | 65.57 ± 10.07* | 91.08 ± 25.29 | — | 112.50 ± 15.00 | 78.27 ± 15.90* |
| 25 | 104.33 ± 20.14 | 66.86 ± 14.61* | 89.50 ± 19.87 | 84.80 ± 5.27* | 118.13 ± 15.80 | 68.64 ± 8.97* |
| 30 | 118.33 ± 29.53 | 60.14 ± 15.85* | 85.30 ± 19.64 | 74.20 ± 6.65* | 113.13 ± 14.98 | 70.18 ± 11.15* |

* Significant at 95% confidence interval.

these two sets of experiments are close to each other. The reconstitution experiments also reveal the following (ref. Fig. 1): (i.) The sequence of addition of dialyzed CF or the divalent cations to the system does not alter the extent of clumping after reconstitution as judged from the closeness of points in Figure 1. (ii.) The time course of clumping, for the 15-min period following reconstitution, with reconstituted CF is different from that with normal CF. In reconstitution experiments a lag period was evident when no significant reduction in nonclumped-cell numbers was observed until 15 min after reconstitution. (The two points corresponding to 40 min period in Figure 1 are not significantly different from zero clumping observed at the time of reconstitution.) (iii.) The extent of clumping at the end of 30 min after reconstitution (60 min from time 0) was 7% higher than that with normal CF at 10 $\mu$l/ml during a similar clumping period. This shows that the addition of the equivalent amount of divalent cations to dialyzed CF or *vice versa* restores the clumping effectiveness of dialyzed CF. Furthermore, it indicates a synergism between the divalent cations and the CF. (iv.) Since clumping could be achieved with the addition of 10 $\mu$l/ml of normal CF but not with the same concentration of dialzyed CF or equivalent concentrations of $Ca^{2+}$ and $Mg^{2+}$, it is clear that CF-mediated coelomocyte clumping in our system is due to a factor(s) present in CF. (v.) From the conclusions stated in (iii) and (iv) above, it follows that the clumping factor(s) present in CF requires $Ca^{2+}$ and $Mg^{2+}$ to produce clumping of coelomocytes, and in the absence of these divalent cations the factor(s) is(are) ineffective as a clumping agent(s).

## DISCUSSION

The results indicate that coelomocytes clump when suspended in CMFSS containing clumping concentrations of CF, $Ca^{2+}$, and/or $Mg^{2+}$. The failure of the coelomocytes to clump when suspended in CMFSS, or in CMFSS containing CF below 7.5 $\mu$l/ml (Table II), 0.23 m$M$ $Ca^{2+}$, 0.50 m$M$ $Mg^{2+}$, or 0.1 m$M$ $Ca^{2+}$ + 0.6 m$M$ $Mg^{2+}$ (Table III), demonstrates that: (i) the clumping of these cells is dependent on the presence of $Ca^{2+}$ and/or $Mg^{2+}$ and a clumping factor(s) in the suspending medium; and (ii) a minimum concentration of $Ca^{2+}$ and/or $Mg^{2+}$ in the medium is necessary for clumping to occur. The necessary concentration of $Ca^{2+}$ and/or $Mg^{2+}$ for clumping is also dependent on whether or not the ions are used with CF, and whether they are used separately or in combination.

Since the agglutination of hemostatic cells in many animals, including mammalian platelets, is dependent on the presence of $Ca^{2+}$ and $Mg^{2+}$ in the medium (see review by Belamarich, 1976; Massini, 1977), it is not surprising to find that the coelomocytes of *A. forbesi* require these ions for clumping *in vitro*. However, the finding of Boolootian and Giese (1959) that clumping of the coelomocytes of 8 species of sea stars (which do not include *A. forbesi*) is independent of $Ca^{2+}$ warrants critical examination in the light of the present results. The authors drew this conclusion because, in their system, sea star coelomocytes clumped in the presence of EDTA. In our collection system, which also contained EDTA, the coelomocytes remained separate and nonclumped. The species difference, while it could be a factor, is an unlikely explanation for this difference in results.

Thus the reason for coelomocyte clumping in the collection system of Boolootian and Giese must be sought in the technique used by the authors rather than in the species difference. They collected 0.9 ml of coelomic fluid in 0.1 ml of EDTA solution and used 2 m$M$ and 13 m$M$ EDTA at pH 7.6 in their final collection mixture. We used EDTA at a final concentration of 12.75 m$M$ at pH 7.4. It is unlikely that the pH difference would account for the diametrically opposite results obtained. However, it is possible that a certain amount of $Ca^{2+}$ and/or $Mg^{2+}$ was left unchelated in the system, and these free ions might have produced clumping. Thus, it becomes essential to discuss the kinetics of chelation of these divalent cations with EDTA in the system used by Boolootian and Giese.

The amount of EDTA present in the total 1 ml mixture of Boolootian and Giese was either $2 \times 10^{-3}$ or $13 \times 10^{-3}$ mmoles. The authors did not report the concentrations of $Ca^{2+}$ and $Mg^{2+}$ in the coelomic fluid of the sea stars they used. Thus, for purposes of present calculations, we have used the data reported by Binyon (1972) which show that the average concentrations of $Ca^{2+}$ and $Mg^{2+}$ in the coelomic fluid of four species of sea stars (*Astropecten* sp., *Solaster endica*, *Asterias vulgaris*, and *Marthaster glacialis*) are 10.8 m*M* and 46.4 m*M*, respectively. Accordingly, the respective amounts of these ions present in the coelomic fluid-EDTA mixture of Boolootian and Giese were $9.7 \times 10^{-3}$ mmoles of $Ca^{2+}$ and $41.8 \times 10^{-3}$ mmoles of $Mg^{2+}$. Although the amounts of free $Ca^{2+}$ and $Mg^{2+}$ in the coelomic fluid would be less than the total amounts (because of association with other ions), it is reasonable to use the figures for the total amounts of these ions in calculations of their chelation with EDTA. The formation constants for Ca-EDTA and Mg-EDTA at pH 7 are $2.5 \times 10^7$ and $2.5 \times 10^5$, respectively (Kolthoff et al., 1969). Therefore, when both ion species are present in an equal mole ratio, Ca-EDTA is expected to be formed preferentially over Mg-EDTA in a ratio of 100:1. However, in the above coelomic fluid mixture the mole ratio of $Ca^{2+}:Mg^{2+} = 1:4.3$. This would produce Ca-EDTA and Mg-EDTA in an approximate ratio of 23:1. Since one mole of EDTA binds one mole of divalent cation, it follows that the maximum amount of divalent cation-EDTA complex that could be formed in the mixture is either $2 \times 10^{-3}$ mmoles or $13 \times 10^{-3}$ mmoles, depending on the concentration of EDTA used.

With a binding ratio of 23:1 and with $9.7 \times 10^{-3}$ mmoles of $Ca^{2+}$ present in the mixture, it can be easily calculated that $2 \times 10^{-3}$ mmoles of EDTA could bind only $1.91 \times 10^{-3}$ mmoles of $Ca^{2+}$. Therefore, $7.8 \times 10^{-3}$ mmoles of $Ca^{2+}$ and all the $Mg^{2+}$ would be left uncomplexed in the mixture. However, with 13 m*M* EDTA, all $Ca^{2+}$ present in the mixture would be chelated, while $38.5 \times 10^{-3}$ mmoles of $Mg^{2+}$ would be left uncomplexed. The concentration of this uncomplexed $Mg^{2+}$ in the mixture is about 51× more than the clumping concentration (0.75 m*M*) reported here. Obviously, when the system contained 2 m*M* EDTA, uncomplexed $Ca^{2+}$ had produced clumping. When it contained 13 m*M* EDTA, uncomplexed $Mg^{2+}$ was probably responsible for clump induction. Further, our data show that $Mg^{2+}$, in the absence of $Ca^{2+}$, is not capable of maintaining clumps, provided the cell suspension in anticoagulant solution is stirred properly. Collecting coelomic fluid from the animals in a syringe, as was done by Boolootian and Giese, probably did not provide sufficient mixing of the fluid. This insufficient mixing together with uncomplexed $Ca^{2+}$ or $Mg^{2+}$ produced clumping in their collection system. Hence, their characterization that the clumping of sea star coelomocytes is independent of $Ca^{2+}$ is unwarranted. The present findings and those of Jangoux and Vanden Bossche (1975) clearly demonstrate that the clumping of the sea star coelomocytes depends on the presence of $Ca^{2+}$ and/or $Mg^{2+}$ in the medium.

### *Clumping pattern with CF, $Ca^{2+}$ and/or $Mg^{2+}$*

The time course of clumping in the presence of CF at 10 μl/ml, 0.23 m*M* $Ca^{2+}$ + 0.12 m*M* $Mg^{2+}$, or 0.45 m*M* $Ca^{2+}$ is biphasic with an initial phase occurring during the first 5-min period and a second phase following (Fig. 1). This biphasic mode of clumping, however, was not evident in two cases. The second phase clumping was absent with CF concentration of 7.5 μl/ml, while no initial phase could be discerned with 0.75 m$M$ $Mg^{2+}$ (Fig. 1).

Further, the data presented in Figure 1 reveal that the degree of clumping in the initial phase was variable and increased from 19% with CF to 33% with $Ca^{2+}$ and $Ca^{2+}$ + $Mg^{2+}$ (except with 0.75 m$M$ $Mg^{2+}$). Evidently not all coelomocytes that were potentially capable of clumping formed clumps with CF concentration of 7.5 or 10 μl/ml. It is interesting to note that the two clumping concentrations of CF produced identical clumping in the initial phase. Similarly, 0.45 m$M$ $Ca^{2+}$ and 0.23 m$M$ $Ca^{2+}$ + 0.12 m$M$ $Mg^{2+}$ also produced identical clumping in the initial phase (Fig. 1) even though they differed in their $Ca^{2+}$ concentrations by a factor of 2. Since $Mg^{2+}$ potentiates the clumping action of $Ca^{2+}$, a lower $Ca^{2+}$ concentration in the presence of $Mg^{2+}$ could produce clumping identical to that with a higher $Ca^{2+}$ concentration in the absence of $Mg^{2+}$. Identical clumping rates observed with 0.23 m$M$ $Ca^{2+}$ + 0.12 m$M$ $Mg^{2+}$ and 0.45 m$M$ $Ca^{2+}$ might be coincidental and not necessarily indicative of maximal clumping for the initial phase. Variations in cell counts among replicate experiments which resulted in abolition of the initial phase in the case of 0.75 m$M$ $Mg^{2+}$ could have occurred if the individual cells forming clumps were not adhered to each other firmly. The "loose" clumps would dissociate easily and produce large variations in nonclumped-cell counts. This implies that with 0.75 m$M$ $Mg^{2+}$ it takes longer for the coelomocytes to develop "stickiness" and, therefore, more time is required to form "tight" clumps *in vitro.*

Sponge cells suspended in solutions containing EDTA suffer some damage and are inhibited from clumping, and this effect can be reversed by supplying proper amounts of $Ca^{2+}$ and/or $Mg^{2+}$ (Humphreys, 1963). EDTA inhibition of *Limulus* amoebocyte aggregation was reversed completely by adding 24 m$M$ $Mg^{2+}$ or *Limulus* "serum" at 1:20 dilution, but reversal was incomplete with 32 m$M$ $Ca^{2+}$ (Kenney *et al.*, 1972). Our results show that coelomocytes of *A. forbesi,* which have been inhibited from clumping with EDTA during collection and centrifugation, resume their clumping activity at a faster pace if immediately suspended in CMFSS containing clumping concentrations of CF, $Ca^{2+}$ + $Mg^{2+}$, or $Ca^{2+}$ than they do if immediately suspended in CMFSS containing a clumping concentration of $Mg^{2+}$.

During the second phase, although the overall extent of clumping increased in a linear fashion during a 25-min period, the rate of clumping was slower than that in the initial phase. Further, CF concentration of 10 μl/ml, 0.23 m$M$ $Ca^{2+}$ + 0.12 m$M$ $Mg^{2+}$, and 0.45 m$M$ $Ca^{2+}$ produced a clumping rate of 6% per 10 min in the second phase, suggesting that the second phase clumping occurs independently of the initial phase. This conclusion is also supported by the results with CF at a concentration of 7.5 μl/ml which did not show any significant clumping in the second phase while producing a 19% clumping in the initial phase. In addition, a constant clumping rate during the second phase in contrast to variable rates in the initial phase suggests that the mechanism for coelomocyte clumping are different for each phase.

### *Clumping lag with reconstituted CF*

The delay in initial clumping in reconstitution experiments could have been produced by the prolonged stay of the coelomocytes in dialyzed CF or nonclumping concentrations of $Ca^{2+}$ + $Mg^{2+}$. Had EDTA produced any damage to the coelomocytes during collecting and centrifugation it could only have been accentuated by not returning the cells to a medium containing proper amounts of $Ca^{2+}$

and/or $Mg^{2+}$. Consequently, prolonged inhibition of clumping under these conditions would require longer recovery time and produce a lengthy lag period before clumping. This reasoning, while it explains the delay in initial clumping, also implies that clumping is brought about by the "stickiness" of the coelomocytes and that a nonclumping environment impairs the development of this "stickiness."

That the divalent cation chelators, EDTA and EGTA, may affect cell adhesion by removing materials from cell surfaces has been postulated for different cell types (Weiss, 1960; Curtis, 1973; Moscona, 1973). Nobel (1970) has also expressed similar views with regard to the effects of EDTA at pH 6.0 on the aggregation of the coelomocytes of the sea cucumber *Cucumaria frondosa.*

### *The coelomocyte-clumping factor in CF*

The results presented here strongly suggest the existence of a coelomocyte clumping factor in CF which requires $Ca^{2+}$ and $Mg^{2+}$ to be effective. That the factor is nondialyzable and heat labile is also indicated by the results. It might be argued that dialyzing CF against CMFSS had removed not only $Ca^{2+}$ and $Mg^{2+}$ but also other constituents, such as trace elements and low molecular weight organic compounds, from CF. The removal of these other substances could affect clumping. Although the argument is reasonable, it is unlikely that these components exerted any effect on clumping of the coelomocytes. The restoration of clumping with reconstituted CF would not have been possible if components other than $Ca^{2+}$ and $Mg^{2+}$ had any appreciable effect on clumping.

### *Source and nature of clumping factor(s)*

Two possible sources of the clumping factor(s) exist. The factor(s) could have been released (i) by the injured tissue, and/or (ii) from the coelomocytes during collection of the coelomic fluid and the preparation of the CF. Extracts of echinoderm tissues have been shown to cause coelomocyte clumping (Donnellon, 1938; Bookhout and Greenberg, 1940; Davidson, 1953; Boolootian and Giese, 1959). That an extract prepared from coelomocytes can mediate coelomocyte clumping in the sea star has also been demonstrated (Bang and Lemma, 1962; Bang, 1970). The latter observation has gained strength by the isolation and characterization of a clumping factor from the coelomocytes of *A. forbesi* (Prendergast and Suzuki, 1970; Prendergast and Liu, 1976). According to these authors, the factor is a basic protein with a molecular weight of approximately 38,000 daltons. The nondialyzability and the heat labile nature of the factor(s) reported here would also indicate that it (they) is (are) a protein(s). However, further experimental work is needed to determine the exact nature and the source of the clumping factor(s).

### *Mechanism of cellular clotting*

Clotting of the coelomic fluid in echinoderms is achieved by the agglutination of the coelomocytes. Thus, an *in vitro* analysis of the mechanism of cellular aggregation (agglutination or clumping) is equated with the analysis of the mechanism of clotting.

Since Geddes (1880) first documented the cellular clotting in echinoderms many investigators have provided useful information on the subject (see review by Kanungo, 1982). However, except for the work of Boolootian and Giese (1959), none of the reports provide experimental studies on the clotting itself. The coelomocytes of all echinoderms form clumps *in vitro* (Endean, 1966; Johnson and Beeson, 1966;

Johnson, 1969; Bang, 1970; Chien *et al.*, 1970; Noble, 1970; Fontaine and Lambert, 1977; Bertheussen and Seljelid, 1978; Kaneshiro and Karp, 1980; Kanungo, 1982). In asteroid echinoderms, the predominant type of coelomocyte is the bladder amoebocyte, which takes part in clotting (Johnson and Beeson, 1966; Bang, 1970; Kanungo, 1982). Transformation of bladder to filiform amoebocytes, which was thought to be a prerequisite for cellular clotting in sea stars (Boolootian and Giese, 1958, 1959), has since been disputed by Johnson and Beeson (1966) and Kanungo (1982). Also, the contention of Boolootian and Giese (1959) that the cellular clotting in asteroid echinoderms does not require $Ca^{2+}$ is no longer tenable in the light of the present findings and those of Jangoux and Vanden Bossche (1975).

Our results clearly demonstrate the requirement of $Ca^{2+}$ and/or $Mg^{2+}$ for clumping of the coelomocytes *in vitro*. Further, it is also shown that in the presence of CF (which contains the clumping factor) the requirement for these divalent cations for cellular clumping *in vitro* is less than it is without CF. Taken together these findings suggest that the clumping factor(s) alters the permeability of the cell membrane to divalent cations in a way which increases the influx of these ions. As a result, the intracellular concentrations of $Ca^{2+}$ and $Mg^{2+}$ increase to levels at which clumping becomes possible. In other words, the coelomocyte clumping depends on the intracellular rather than the extracellular concentrations of these ions. This is not unusual in light of the second messenger role played by $Ca^{2+}$ in coordinating diverse cellular activities in many cell types (Rasmussen, 1970; Berridge, 1975), including mammalian platelets (Massini, 1977). The above hypothesis also predicts that in the absence of the clumping factor, a higher concentration of $Ca^{2+}$ and/or $Mg^{2+}$ in the medium would be required to establish a concentration gradient that would favor a greater influx of these ions which in turn would cause clumping. The present results substantiate this hypothesis because clumps could be formed with CF concentration at 7.5 $\mu$l/ml which contains 0.075 m*M* $Ca^{2+}$ and 0.45 m*M* $Mg^{2+}$, but in the absence of CF clumping could not be effected even at concentrations of 0.1 m*M* $Ca^{2+}$ + 0.6 m*M* $Mg^{2+}$.

### *Biphasic clumping and its implications*

A two-stage coelomocyte clumping has been reported in the holothurian, *Cucumaria frondosa*, by Fontaine and Lambert (1977). The authors contended that the initial fast aggregating stage was brought about by the transitional cells which were present in the coelomic fluid before it was withdrawn from the animal, but the second slow phase was due to the transformation of the bladder amoebocytes to the transitional form which occurs at a slower pace *in vitro*.

It is, therefore, conceivable that the biphasic clumping reported here is due to two functional cell populations (a fast reacting population and a slow reacting one) which exist among the coelomocytes of *A. forbesi*.

The cells in the two groups probably differ in their permeability characteristics and their ability to establish surface properties for clumping. The fast reacting cells become "sticky" faster than the slow ones in the presence of a clumping stimulus. Whether these two populations of coelomocytes differ in their morphological characteristics is not known. Current investigations on intercellular adhesion implicate cell surface glycoproteins (Roseman, 1974; Oppenheimer, 1977, 1979) and lectins (Brown and Hunt, 1978; Rosen and Kaur, 1979) in generating sites for mutual adhesion of cells in a variety of cells systems. Similar studies using the coelomocytes of echinoderms would provide useful information for elucidating the mechanism of cellular clumping in these animals.

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# MALE PRONUCLEAR DEVELOPMENT IN STARFISH OOCYTES TREATED WITH 1-METHYLADENINE

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## ABSTRACT

Light and electron microscopic observations were carried out in order to examine the relation between male pronuclear development and the state of "cytoplasmic maturation" acquired by starfish oocytes under the influence of 1-methyladenine (1-MA). Alterations were not apparent in the germinal vesicles or incorporated sperm nuclei of inseminated immature *Asterias* eggs for up to 5 hours in the absence of 1-MA. With the addition of 1-MA dramatic changes occurred in the germinal vesicle and ooplasmic region associated with incorporated sperm nuclei. These were followed by alterations in the sperm nucleus leading to the development of a male pronucleus. Pronuclear development in *Asterias* eggs inseminated at the germinal vesicle stage and then treated with 1-MA differed from that described for other organisms. Aside from the dilation of its perinuclear cisterna, the sperm nuclear envelope persisted intact throughout development. Dispersion of condensed chromatin occurred simultaneously throughout the whole of the sperm nucleus. These results suggest that factors necessary for pronuclear development do not exist in the ooplasm of immature starfish oocytes but arise following dispersal of germinal vesicle contents into the cytoplasm.

## INTRODUCTION

The eggs of most animals initiate meiotic maturation prior to ovulation, become arrested at a specific stage of meiosis, and resume maturation after insemination. Although eggs may be experimentally manipulated to ferilize prematurely, *i.e.*, at an earlier stage of meiosis, investigations with the ova of a number of different organisms have indicated that germinal vesicle breakdown is a prerequisite in establishing a condition of cytoplasmic maturation which supports the transformation of a fertilizing spermatozoon into a male pronucleus (Skoblina, 1974, 1976; Hirai, 1976; Katagiri and Moriya, 1976; Thadani, 1979; Balakier and Tarkowski, 1980; Hirai *et al.*, 1981).

Germinal vesicle-intact (immature) starfish oocytes, induced to mature by ovarian hormone (1-methyladenine; 1-MA), develop normally when fertilized (Kanatani and Shirai, 1967; Schuetz and Biggers, 1967; Kanatani *et al.*, 1969). Germinal vesicle-intact oocytes may also be inseminated and subsequently treated with 1-MA to induce germinal vesicle breakdown (Cayer *et al.*, 1975; Schuetz, 1975; Schuetz and Longo, 1981). That the onset of germinal vesicle breakdown can be controlled by exogenous substances in starfish eggs provides a means of studying nucleocytoplasmic interactions during male pronuclear development and the role of 1-MA in fertilization and the onset of development.

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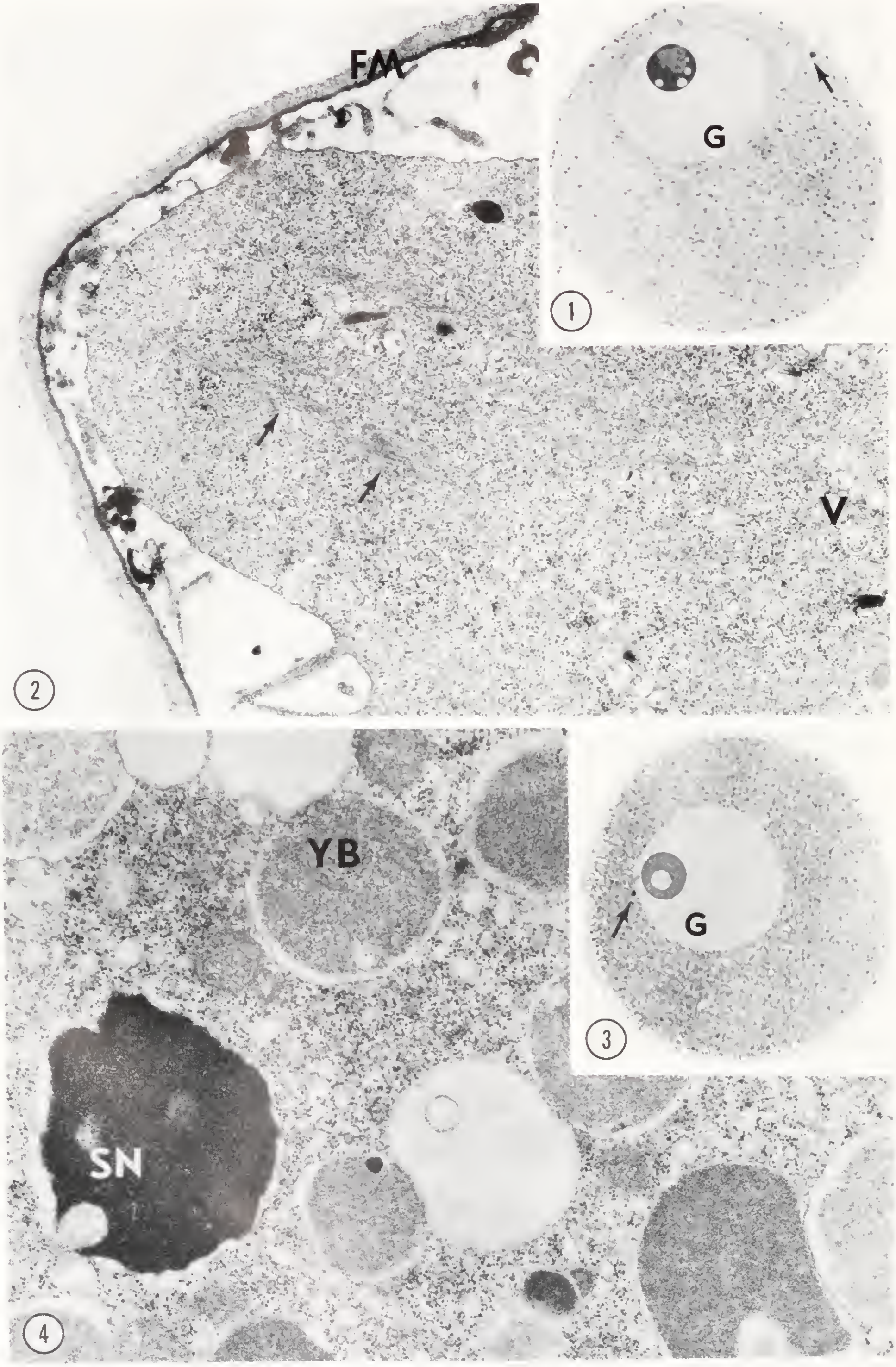
FM
G
1
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The present light and electron microscopic study examines the relation between processes of fertilization and the state of "cytoplasmic maturation" acquired by oocytes under the influence of 1-MA. For this purpose observations were designed to analyze sperm-egg interactions in fertilized, intact germinal vesicle oocytes of the starfish, *Asterias forbesi,* before and after exposure to 1-MA. Brief accounts of these observations have been published previously (Schuetz and Longo, 1979, 1981).

## MATERIALS AND METHODS

Germinal vesicle-intact oocytes were obtained from ripe *Asterias forbesi* as described by Longo *et al.* (1982). Oocytes were fertilized with a 0.1% suspension of sperm and samples were fixed at varying intervals up to 5 hours postinsemination. At forty-five minutes postinsemination a portion of this suspension was treated with 1-MA (1 μg/ml) and samples were taken at varying intervals and prepared for light and electron microscopy as previously described (Longo *et al.*, 1982). A second suspension of oocytes was treated with 1-MA (1 μg/ml) to induce meiotic maturation; these oocytes are referred to as maturing ova. Approximately 30 minutes later the eggs were fertilized and sampled at periodic intervals up to 90 minutes postinsemination.

## RESULTS

Within 5 minutes of gamete mixing, sperm were seen within immature and maturing ova, usually located at the base of the fertilization cone (Fig. 1). By this time the cortical granule reaction was completed and a fertilization membrane surrounded the inseminated egg (Figs. 1, 2). All of the immature starfish oocytes examined in this study were polyspermic (Schuetz and Longo, 1981); eggs that had undergone germinal vesicle breakdown prior to insemination were monospermic. A more detailed ultrastructural account of male and female pronuclear development and association in monospermic, maturing *Asterias* eggs is the subject of a subsequent report; light microscopic observations have been presented by Hirai *et al.* (1981).

The fertilization cone, through which the sperm nucleus passed during its incorporation, was larger in germinal vesicle oocytes than in maturing eggs. When the fertilization cone achieved its maximum dimensions (at about 5 minutes postinsemination) in germinal vesicle-intact eggs it extended approximately 2.5 μm from the oocyte surface and was about 1 μm in diameter at its base. Morphologically it contained a granular substance and fascicles of microfilaments. Along its proximal aspect were numerous vesicles (Fig. 2).

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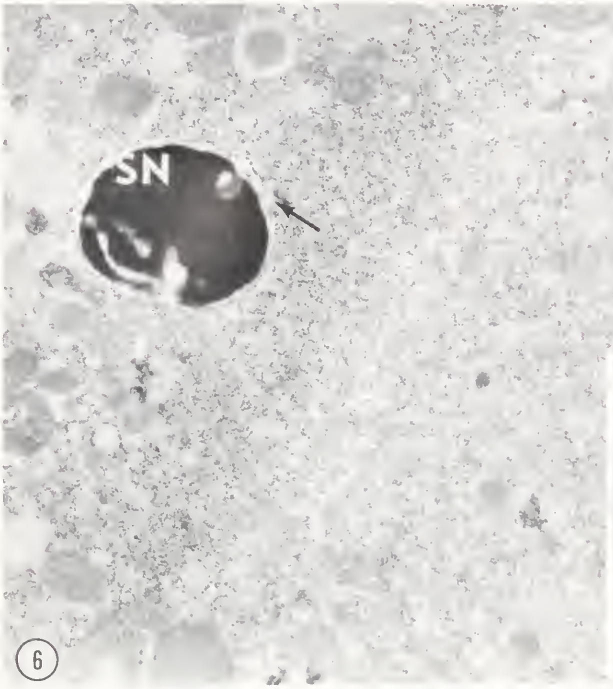
FIGURE 1. Immature *Asterias* oocyte, 5 minutes postinsemination. At the base of the fertilization cone is an incorporated sperm nucleus (arrow). G, germinal vesicle containing a nucleolus. ×1,000.

FIGURE 2. Fertilization cone of an immature oocyte, containing ground substance and fascicles of microfilaments (arrows), 5 minutes postinsemination. Along the base of the fertilization cone are aggregations of vesicles (V). FM, fertilization membrane. ×14,000.

FIGURE 3. Immature oocyte, 30 minutes postinsemination. An incorporated sperm nucleus is depicted at the arrow. The germinal vesicle (G) is structurally similar to those observed in unfertilized oocytes. ×1,200.

FIGURE 4. Sperm nucleus (SN) incorporated into an immature oocyte. In inseminated, immature oocytes, ooplasmic organelles surround the incorporated sperm nucleus and a specialized region, lacking organelles and characteristic of fertilized eggs treated with 1-MA, is not observed. YB, yolk bodies. ×10,000.



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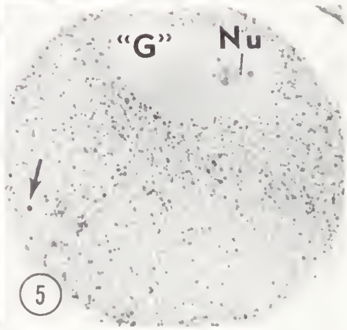

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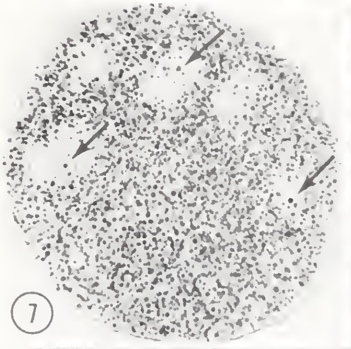

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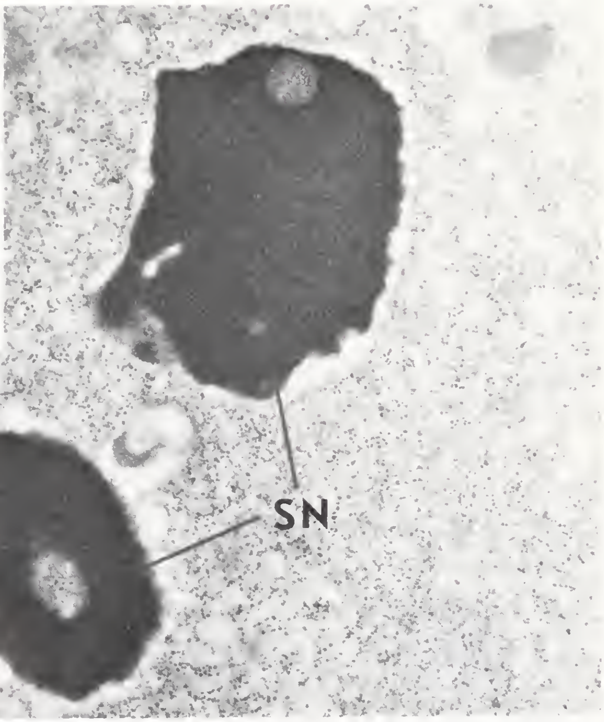

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Sperm nuclei incorporated into germinal vesicle eggs initially were found within the cortex; with time (*i.e.*, by 30 minutes postinsemination) however, they were distributed throughout the cytoplasm without any apparent relation to ooplasmic components (Fig. 3). The cytoplasmic area which surrounded incorporated sperm nuclei was unspecialized in that it contained the same content of organelles and inclusions as observed in other portions of the oocyte (Fig. 4). When inseminated oocytes were maintained at 20°C with gentle agitation, sperm nuclei remained unchanged for up to 5 hours. Throughout this period the sperm nuclear envelope remained intact and the condensed sperm chromatin showed no signs of dispersion (Fig. 4). Moreover, the process of insemination and the presence of incorporated sperm did not appear to have any morphological effect on the germinal vesicle (Fig. 3).

By 15 minutes following the addition of 1-MA profound, structural alterations were apparent in the germinal vesicle, as well as with the ooplasm surrounding incorporated sperm nuclei. The cytoplasmic area surrounding the sperm nucleus became clear of organelles, such as yolk bodies and mitochondria, and within this region accumulated endoplasmic reticulum and ground substance (Figs. 5, 6). This cytoplasmic area enlarged to greater than 12 $\mu$m in diameter before morphological changes were noted within sperm nuclei (Fig. 7). In many polyspermic eggs more than one sperm nucleus was associated with such a specialized area (Fig. 8).

By 30 minutes following the addition of 1-MA, changes in incorporated sperm nuclei had become apparent. Dilation of the perinuclear cisternae was pronounced, and alterations in the density and composition of the condensed chromatin occurred. The actual disruption or removal of the sperm nuclear envelope, similar to that seen in other species (Longo, 1973), was not observed, and how this membranous structure was modified to accompany the expansion of the paternally derived chromatin was not obvious (Fig. 9).

Chromatin dispersion appeared to differ from that described for pronuclear development in zygotes of other species (*cf.* Longo, 1973, 1981). The condensed chromatin gradually transformed from a dense substance to a dispersed, filamentous mass (Figs. 9–12). These changes occurred simultaneously throughout the sperm nucleus except for that material bordering the inner margin of the nuclear envelope (Figs. 9, 11). The condensed chromatin lining the periphery of the sperm nucleus remained unchanged until late in the development of the male pronucleus (60 to 90 minutes after the addition of 1-MA).

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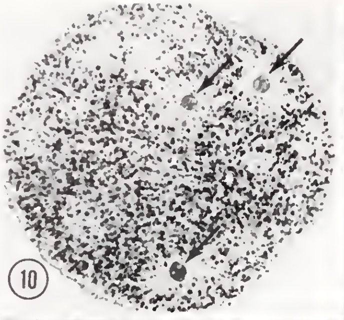
FIGURE 5. Inseminated, immature oocyte, 15 minutes after the addition of 1-MA. The germinal vesicle is breaking down ("G"). An incorporated sperm nucleus, surrounded by a "clear" cytoplasmic area is shown at the arrow. Nu, portion of the disrupting nucleolus. ×1,000.

FIGURE 6. Sperm nucleus (SN) incorporated into an immature oocyte that was subsequently treated with 1-MA (15 minutes after the addition of 1-MA). A cytoplasmic region, relatively devoid of organelles, is associated with the sperm nucleus. The arrow depicts a portion of the sperm nuclear envelope in which the perinuclear cisterna is dilated. ×15,000.

FIGURE 7. Inseminated, immature oocyte subsequently treated with 1-MA. Sperm nuclei which are surrounded by an area relatively free of cytoplasmic organelles are shown at the arrows. Sample fixed 30 minutes after the addition of 1-MA. ×1,000.

FIGURE 8. Two sperm nuclei (SN) of a polyspermic, immature oocyte treated with 1-MA for 15 minutes. Although the condensed chromatin does not show any recognizable changes when compared to oocytes not treated with 1-MA, the perinuclear cisternae of the sperm nuclear envelopes are dilated. ×20,000.



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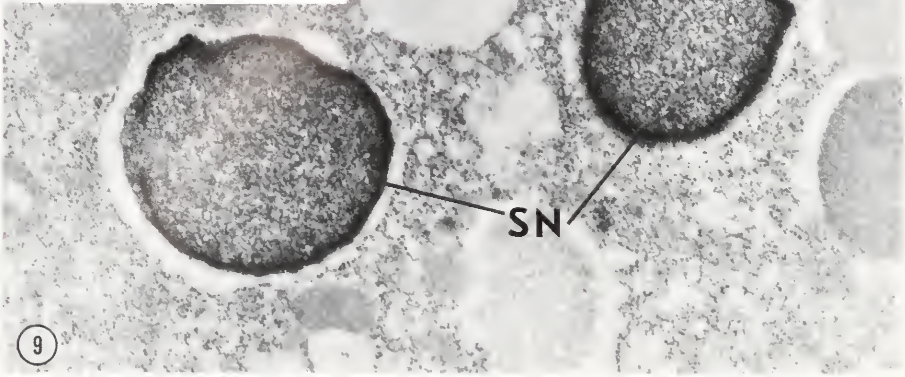

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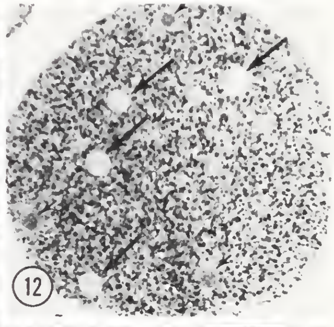

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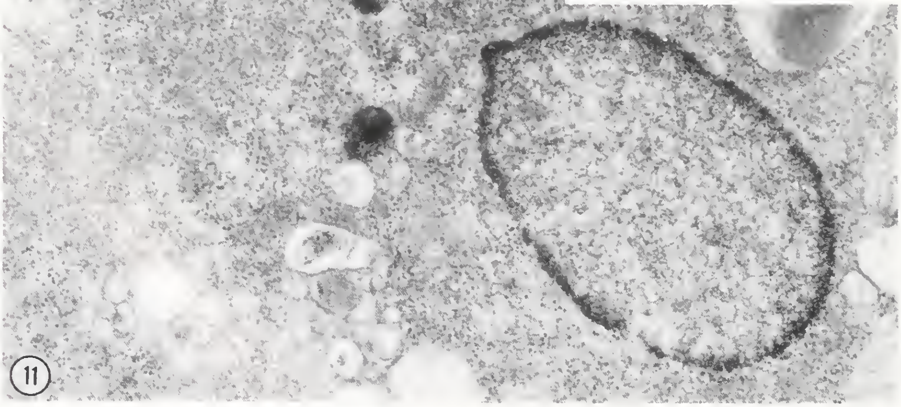

⑪

Changes in sperm nuclear morphology were not uniform as there was considerable asynchrony in pronuclear development in fertilized oocytes treated with 1-MA (Fig. 12). This asynchrony appeared to be a temporal one, since eventually all incorporated sperm nuclei developed into male pronuclei. The relation of this asynchrony to a specific location within the zygote, *e.g.*, the site of germinal vesicle breakdown or sperm entry, was not apparent.

One to 2 hours following the addition of 1-MA, male pronuclei were observed with well-dispersed chromatin and continuous nuclear envelopes (Fig. 13). The nuclear envelope did not demonstrate the dilations of the perinuclear cisternae characteristic of metamorphosing incorporated sperm nuclei. Internally, clear areas, surrounded by a granular nucleoplasm, filled the male pronucleus. Nucleoli composed of a dense granular material also appeared within developed male pronuclei. The male pronuclei continued to enlarge and by 120 minutes following the initiation of pronuclear development measured 5 to 10 $\mu$m in diameter. Large, irregular male pronuclei were also observed within polyspermic zygotes suggesting that the pronuclei fused with one another (Fig. 14).

Following the completion of male pronuclear development the cytoplasmic areas, characteristically associated with transforming sperm nuclei, were greatly reduced in size relative to the size of the male pronucleus. Male pronuclei were surrounded by cytoplasmic areas containing ground substance and some endoplasmic reticulum. This morphology persisted for approximately 2.5 hours after the addition of 1-MA, at which time the pronuclei demonstrated changes characteristic of prophase, *i.e.*, chromosome condensation and nuclear envelope breakdown. Concomitant with these changes spindles were formed in association with the condensing paternally derived chromosomes; the numerous mitotic figures that were produced were observed throughout the fertilized egg (Fig. 15). Of the inseminated immature oocytes treated with 1-MA for 4 hours, less than 10% cleaved into what appeared to be "normal" embryos. Most underwent a succession of divisions such that "morula"-like structures, consisting of blastomeres of different sizes, were produced (Fig. 16).

## DISCUSSION

The microscopic observations presented here document changes induced by 1-MA treatment on sperm nuclei incorporated into immature *Asterias* eggs. Morphological changes in the germinal vesicle or incorporated sperm nuclei were not apparent for up to 5 h in the absence of 1-MA. With the addition of 1-MA dramatic

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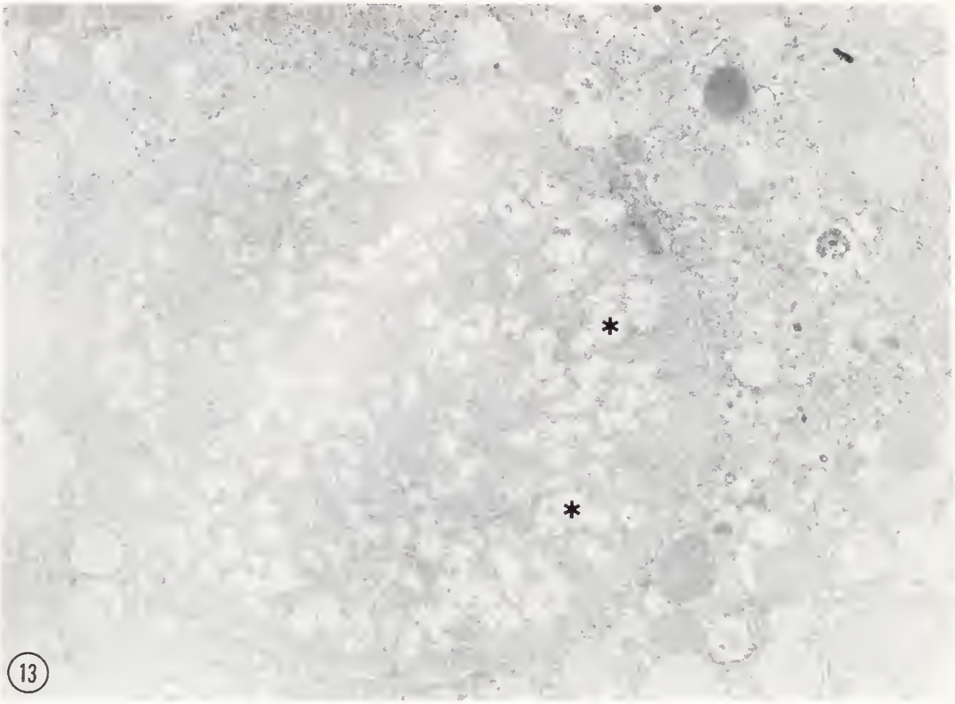
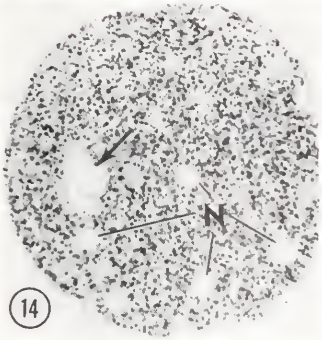
FIGURE 9. Sperm nuclei (SN) of an immature oocyte treated with 1-MA for 30 minutes. The condensed sperm chromatin is dispersing except for that which lines the nuclear envelope. ×28,000.

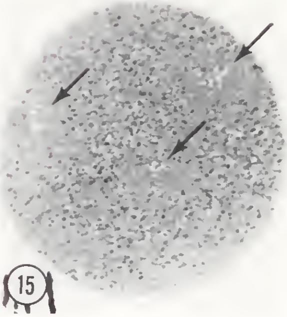
FIGURE 10. Transforming sperm nuclei (arrows) in an immature oocyte treated with 1-MA for 45 minutes. Around each of the developing pronuclei is a specialized cytoplasmic region lacking organelles. ×1,300.

FIGURE 11. Transforming sperm nucleus in an immature oocyte treated with 1-MA for 45 minutes. The condensed chromatin is dispersed except for that located along the periphery of the transforming sperm nucleus. ×29,000.

FIGURE 12. Transforming sperm nuclei at early (small arrows) and later (large arrows) stages of pronuclear development. The earlier stages are distinguished by dense chromatin. Notice that the more developed male pronuclei, *i.e.*, those with the more dispersed chromatin, lack the specialized cytoplasmic areas characteristic of earlier stages. ×1,200.







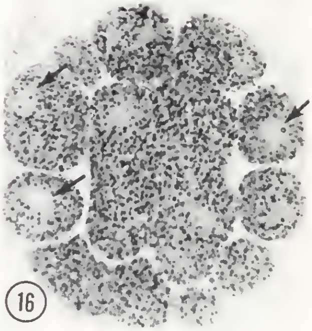


FIGURE 13. Male pronucleus in a fertilized oocyte treated with 1-MA for 60 minutes. A nuclear envelope defines the border of the pronucleus which is surrounded by cytoplasm containing organelles. The clear areas within the pronucleus (*) containing some filamentous material represent areas of chromatin that were extracted by the preparative methods employed. ×14,000.

FIGURE 14. Multiple male pronuclei (N) in a polyspermic oocyte 120 minutes after the addition of 1-MA. The larger, irregular nuclear mass at the arrow appears to be derived from the fusion of a number of pronuclei. The specialized cytoplasmic areas associated with the pronuclei are greatly reduced in size relative to those observed in earlier specimens. ×1,300.

FIGURE 15. Chromosomal masses (arrows) associated with developing spindles and derived from male pronuclei. Specimen prepared 150 minutes after the addition of 1-MA. ×1,200.

FIGURE 16. Inseminated, immature oocyte that has undergone multiple cleavages to form a "morula"-like structure (incubated with 1-MA for 4 hours). The arrows point to nuclei of the blastomeres. ×1,000.

TABLE I

*Temporal relation of meiotic maturation, sperm aster morphogenesis, and male pronuclear development in fertilized immature* Asterias *oocytes (germinal vesicle intact) subsequently treated with 1-MA.*

| | Time after addition of 1-MA (minutes) | | | |
|---|---|---|---|---|
| Process | 0 | 15 | 30 | 60–120 |
| Meiosis* | Intact germinal vesicle | Initiation of germinal vesicle breakdown (plication of surface of germinal vesicle) | Germinal vesicle breakdown (disappearance of nuclear envelope and nucleolus) | Development of meiotic spindle and polar body formation |
| Sperm aster morhogenesis | Not present | Present | Increase in size | Relative reduction in size |
| Sperm nucleus transformation | Sperm nucleus unchanged | Sperm nucleus unchanged | Dilation of sperm nuclear envelope, chromatin decondensation | Completed male pronucleus |

* Taken from Longo *et al.* (1982).

changes were first noted in the germinal vesicle and cytoplasmic region associated with the sperm nucleus. These were followed by alterations in the sperm nucleus leading to the formation of a male pronucleus. Similar results, at the light microscopic level of observation with oocytes of *Asterina pectinifera*, have been reported (Hirai *et al.*, 1981).

The structural reorganization of the cytoplasmic area in association with the sperm nucleus was unexpected as morphologically similar specializations, *e.g.*, asters, are usually preceded by the initiation of pronuclear development in the zygotes of other organisms studied to date (Longo, 1973). The observation that these regions developed only in conjunction with incorporated sperm nuclei suggests that a sperm-derived component (*e.g.*, centrioles) is involved in their formation, possibly as an organizing center. The association of these specialized cytoplasmic regions with sperm nuclei and the fact that they are reminiscent of structures earlier microscopists (Wilson, 1925; *cf.* Hirai *et al.*, 1981) referred to as sperm asters, prompts us to refer to them in a similar manner.

The relation of asters and their development to germinal vesicle breakdown is controversial (*cf.* Masui and Clarke, 1979). The absence of asters in fertilized immature starfish eggs and their development in association with 1-MA-induced meiotic maturation as demonstrated herein suggests that aster formation is related to germinal vesicle breakdown (*cf.* also Franklin, 1965; Longo, 1978) and is supported by investigations in which cellular components, such as basal bodies, initiate the development of asters when injected into mature but not immature amphibian eggs (Heidemann and Kirschner, 1975). On the other hand, enucleation experiments with amphibian eggs have suggested that aster formation is independent of a contribution of germinal vesicle materials (Katagiri, 1974; Skoblina, 1974, 1976).

This and previous studies implicate the germinal vesicle as a source of factors necessary for the transformation of the spermatozoon into a male pronucleus in starfish (Hirai, 1976; Hirai *et al.*, 1981; Schuetz and Longo, 1981). Similar results indicating the control of nuclear activity via factors that arise from or appear in concert with germinal vesicle breakdown have been described (Dettlaff *et al.*, 1964; Niwa and Chang, 1975; Usui and Yanagimachi, 1976; Longo, 1978; Balakier and Tarkowski, 1980; Hylander *et al.*, 1981). The failure of male pronuclear development in enucleate amphibian eggs supports this speculation (Katagiri and Moriya, 1976; Skoblina, 1976). Whether this requirement is the result of specific germinal vesicle factors or arises from nucleo-cytoplasmic interactions following germinal vesicle breakdown is unclear (Kishimoto *et al.*, 1981).

Although the present study of male pronuclear development in *Asterias* employed polyspermic, immature oocytes, it is noteworthy that the transformation of the sperm nucleus in this particular system differed from that described for other species (Longo, 1973). Such differences include the retention of the sperm nuclear envelope, the simultaneous dispersion of chromatin throughout the sperm nucleus, and the formation of the male pronuclear envelope. Changes in the sperm nuclear envelope were not readily apparent in fertilized, immature *Asterias* eggs treated with 1-MA. This membranous structure did not appear to break down by a process of vesiculation as demonstrated in zygotes of many species examined thus far (Longo, 1973, 1981). Aside from the dilation of its perinuclear cisterna, the sperm nuclear envelope persisted intact throughout pronuclear development. This and the dramatic increase in nuclear volume during pronuclear development raises questions as to how the membrane comprising the nuclear envelope is augmented to accommodate the increase in chromatin dispersion. The dilated perinuclear cisternae characteristic of sperm nuclei within *Asterias* oocytes treated with 1-MA may be a manifestation of this augmentation. In addition, vesicles were occasionally observed adjacent to the surface of the developing male pronucleus (F. J. Longo, personal observations). Although we have not been able to document such an event, it is possible that these vesicles fuse with and augment the existing sperm nuclear envelope.

Dispersion of the condensed sperm chromatin in *Asterias* oocytes differed from that described for sea urchins, where decondensation was initiated along the periphery of the sperm nucleus and progressively appeared more centrad. The pattern observed in *Asterias*, *i.e.*, where dispersion occurred simultaneously throughout the whole of the sperm nucleus, is similar to that described for *Barnea*, *Gallus*, and the hamster (Pasteels, 1963; Okamura and Nishiyama, 1978; Longo and So, 1982).

It has been suggested that the asynchrony in pronuclear development, characteristic of polyspermic *Asterias* oocytes treated with 1-MA, may be related to the proximity of sperm nuclei with the germinal vesicle (Schuetz and Longo, 1981). This spacial relation may be involved, but the observations made during the course of this study, where the extent of pronuclear development was not always correlated with the site of germinal vesicle breakdown, suggest that other factors may have a bearing as well. Previous investigations with mammalian eggs have shown that the degree of polyspermy has a profound influence on pronuclear development (Hunter, 1967; Hirao and Yanagimachi, 1979; Witkowska, 1981). In these studies the number of sperm developing into male pronuclei was inversely related to the degree of polyspermy; some sperm nuclei metamorphosed into male pronuclei while the remainder were delayed at an earlier stage of pronuclear development. These results suggest that in polyspermic eggs competition occurs among sperm nuclei for materials responsible for male pronuclear development. That eventually all sperm nuclei develop into male pronuclei in *Asterias* eggs indicates that the inhibition of pronuclear development is not complete but rather a slowing down of sperm nuclear transformations.

Samples of inseminated oocytes subsequently treated with 1-MA and examined just prior to cleavage, contained large nuclei which appeared to be brought about by a fusion of the pronuclei. The presence of large irregular-shaped nuclei as shown in Figure 14 is suggestive of such a process; pronuclear fusion has also been described in fertilized, immature *Asterina* oocytes treated with 1-MA (Hirai *et al.*, 1981). Eventually all of the nuclei entered mitosis forming what appeared to be individual spindles. Similar results have also been reported for the eggs of other organisms (*cf.* Wilson, 1925; Elinson, 1977). Presumably as a result of the numerous mitotic apparatuses present, the zygote is induced to undergo multiple cleavages (Rappaport, 1971, 1975), such that a morula-like structure is produced. These embryos fail to give rise to normal larvae; they eventually degenerate due presumably to an unbalanced genome.

The microscopic observations presented here further illustrate some of the complex hormonal, cytoplasmic and nuclear interactions that occur during egg maturation and fertilization in *Asterias* and that proper synchronization of these events is crucial for normal development. The temporal relation of meiosis, sperm aster morphogenesis, and male pronuclear development in fertilized, immature *Asterias forbesi* oocytes subsequently treated with 1-MA is outlined in Table I; a similar relation has been described for *Asterina pectinifera* (Hirai *et al.*, 1981).

## ACKNOWLEDGMENTS

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# MEIOTIC MATURATION AND THE CORTICAL GRANULE REACTION IN STARFISH EGGS

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## ABSTRACT

Correlative light and electron microscopic studies of immature and maturing starfish (*Asterias forbesi*) eggs have been carried out demonstrating (1) morphological alterations attending meiotic maturation induced by 1-methyladenine and (2) the structure of the egg cortex and cortical granule reaction. Because cortical granule components, are structurally recognizable, their fate and relation to the development of the fertilization membrane could be determined. One and possibly more of the cortical granule components become an integral part of the fertilization membrane. Comparison of maturing and immature ova indicate that germinal vesicle-containing oocytes (immature) are capable of undergoing a cortical granule reaction morphologically similar to that of eggs having undergone germinal vesicle breakdown (maturing).

## INTRODUCTION

In the starfish, spawning and oocyte maturation are stimulated by the ovarian hormone, 1-methyladenine (1-MA), which is synthesized by follicle cells (Kanatani *et al.*, 1969). Isolated oocytes undergo germinal vesicle breakdown, shedding of follicular cells, and maturation in response to externally applied 1-MA at micromolar concentrations. Furthermore, application of 1-MA promotes uniform and synchronous maturation, thereby facilitating the study of oocyte maturation, fertilization, and early development. Ultrastructural investigations of starfish oocytes have examined oocyte-follicle cell relationships and surface changes stimulated by 1-MA (Hirai *et al.*, 1971; Rosenberg *et al.*, Schroeder *et al.*, 1979). As far as we are aware, correlative light and electron microscopic studies of germinal vesicle breakdown and meiotic maturation in *Asterias* oocytes treated with 1-MA have not been presented.

In addition, although the fine structure of the cortex of fertilized *Asterias* eggs has been examined (*cf.* Monroy, 1965), ultrastructural analysis of the cortical granule reaction in this organism has not been presented. Stages before, during, and after the cortical granule reaction in the starfish, *Patiria miniata*, have been described (Holland, 1980). In this study Holland (1980) discussed the question of the presence of a hyaline layer in activated starfish oocytes and suggested that observations made with *Patiria* are representative of the cortical granule reaction in other asteroids. The descriptions of the cortical granule reaction in *Asterias* presented herein have been carried out in light of Holland's speculations.

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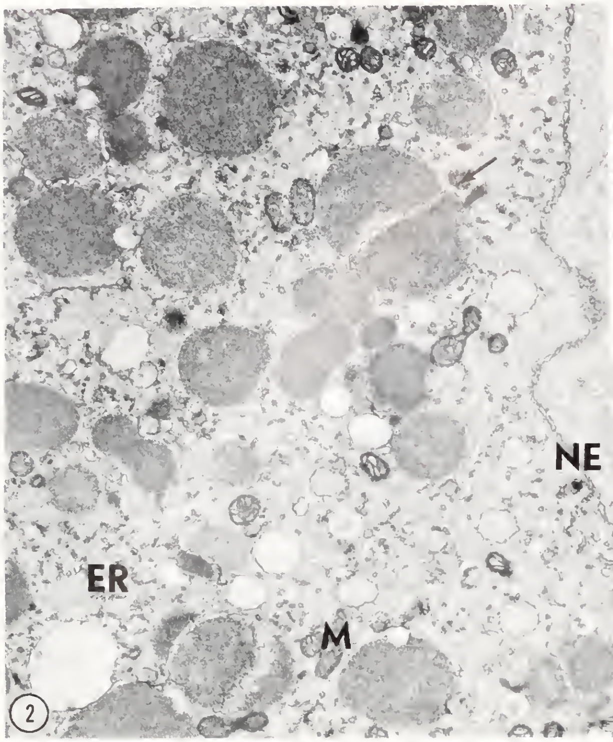
[*] Author to whom correspondence should be addressed (at Univ. of Iowa).



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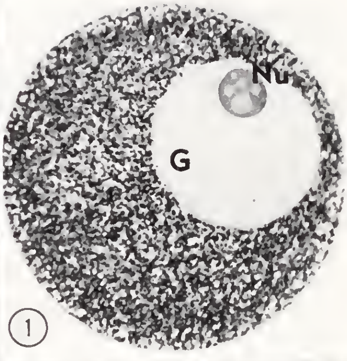

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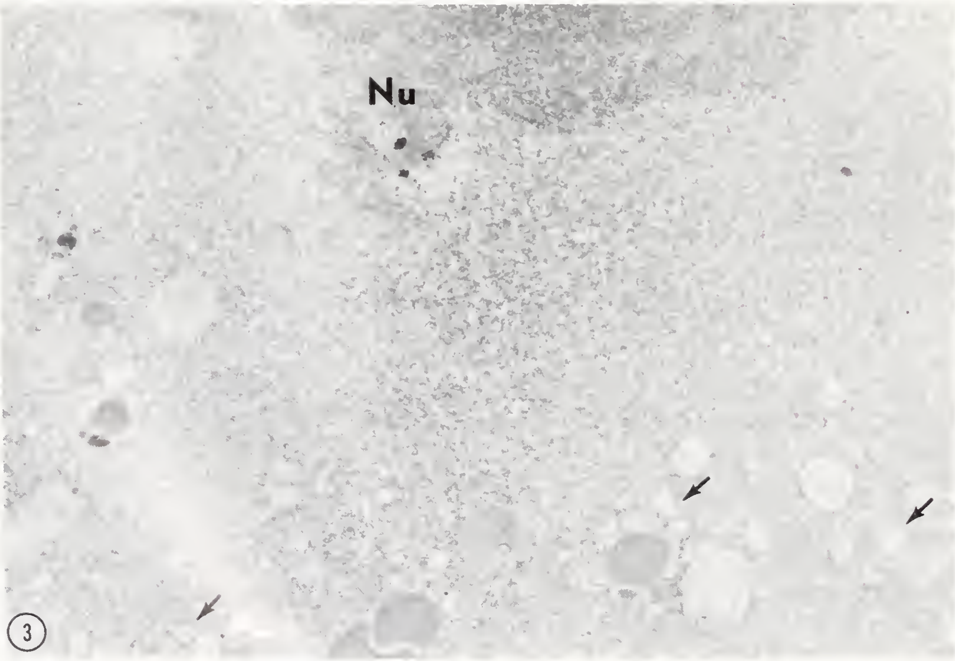

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## MATERIALS AND METHODS

Germinal vesicle-intact oocytes were obtained from ripe starfish (*Asterias forbesi*) ovaries which had been washed previously in calcium-free sea water (CaFSW; Schuetz and Biggers, 1968). Washing in CaFSW inhibited spontaneous nuclear maturation and induced the detachment of follicle cells from the oocytes. Ovaries were minced and germinal vesicle-intact oocytes were separated from follicle cells and returned to artificial sea water containing the normal concentration of calcium (MBL formula; Cloud and Schuetz, 1973). Germinal vesicle breakdown was induced by adding 1-MA (1 $\mu$g/ml; Sigma) to an oocyte suspension, and samples were taken at regular intervals and fixed for 1 hour at 4°C in a solution of sea water containing 2% gluteraldehyde, 0.5% paraformaldehyde, 1% acrolein, 1% sodium citrate, and 4.5% sucrose. The samples were washed overnight in sea water, incubated in 0.5% $OsO_4$ for 30 minutes, dehydrated in ethanol, and embedded in Spurr embedding medium. Manipulation of the specimens during these procedures has been described (Longo and Anderson, 1972). Thick sections were stained with 1% toludine blue and analyzed with a Leitz Orthoplan microscope. Thin sections were stained with uranyl acetate and lead citrate and examined in a Philips 300 electron microscope.

In order to examine the cortical granule reaction, germinal vesicle-intact eggs, induced to mature to the first metaphase of meiosis with 1-MA, and ova collected from spontaneously ovulating females which had undergone germinal vesicle breakdown were inseminated with sperm collected from isolated testes. Just prior to insemination sperm were diluted to 0.1% (v/v) in sea water. Specimens were fixed for 1 hour at 30-second intervals and then at 1-minute intervals for up to 10 minutes post insemination. Further processing was carried out as described above. In this report oocytes containing a germinal vesicle are referred to as "GV-intact" or "immature" ova; oocytes that have undergone germinal vesicle breakdown are referred to as "maturing" eggs.

## RESULTS

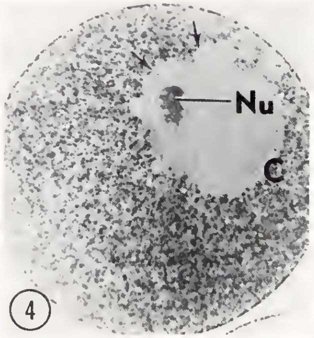
### *Germinal vesicle oocytes*

The germinal vesicle of the *Asterias* oocyte was a large spheroid body, containing a homogeneous nucleoplasm in which was suspended a single nucleolus (Fig. 1). The nucleolus was composed of a dense, fine-textured material containing one or more areas of lesser density. The periphery of the germinal vesicle was delinated by a smooth-surfaced nuclear envelope (Fig. 2). Spheroid yolk bodies, containing a dense homogenous substance, were found in close association with one another; in these instances their juxtaposed surfaces were flattened. Vesicles, some comparable in size to yolk bodies but not nearly as numerous, were observed within the cytoplasm. In addition, smaller vesicles, some with a filamentous material, others lacking

FIGURE 1. *Asterias* oocyte containing a germinal vesicle (G) and nucleolus (Nu) consisting of two structural components. The granularity of the cytoplasm is due to yolk bodies and mitochondria. ×1150.

FIGURE 2. Electron micrograph depicting a portion of the germinal vesicle (G) and adjacent cytoplasm. The yolk bodies are frequently found as aggregates seen at the arrow. M, mitochondria; ER, endoplasmic reticulum; NE, nuclear envelope. ×15,000.

FIGURE 3. Portion of a germinal vesicle initiating the resumption of meiosis (*i.e.*, germinal vesicle breakdown), 30 minutes after exposure to 1-MA. The vesicles depicted by the arrows are presumably products of the vesiculation of the nuclear envelope. Nu, disrupting nucleolus. ×10,000.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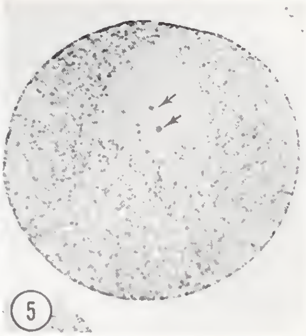
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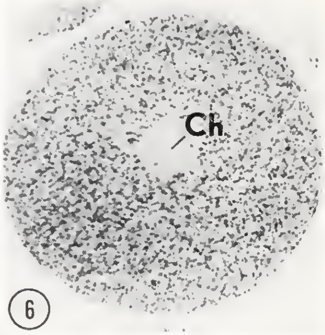
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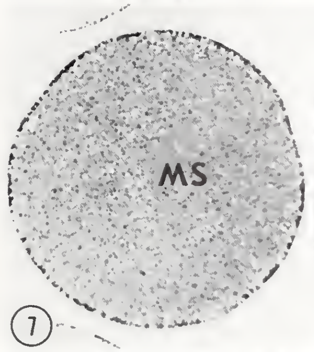
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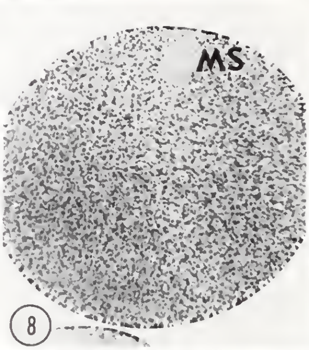
MS
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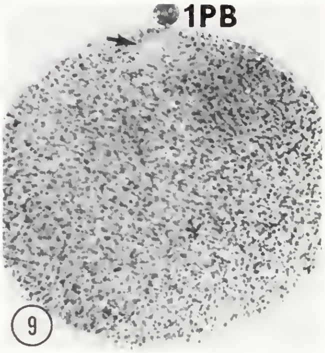
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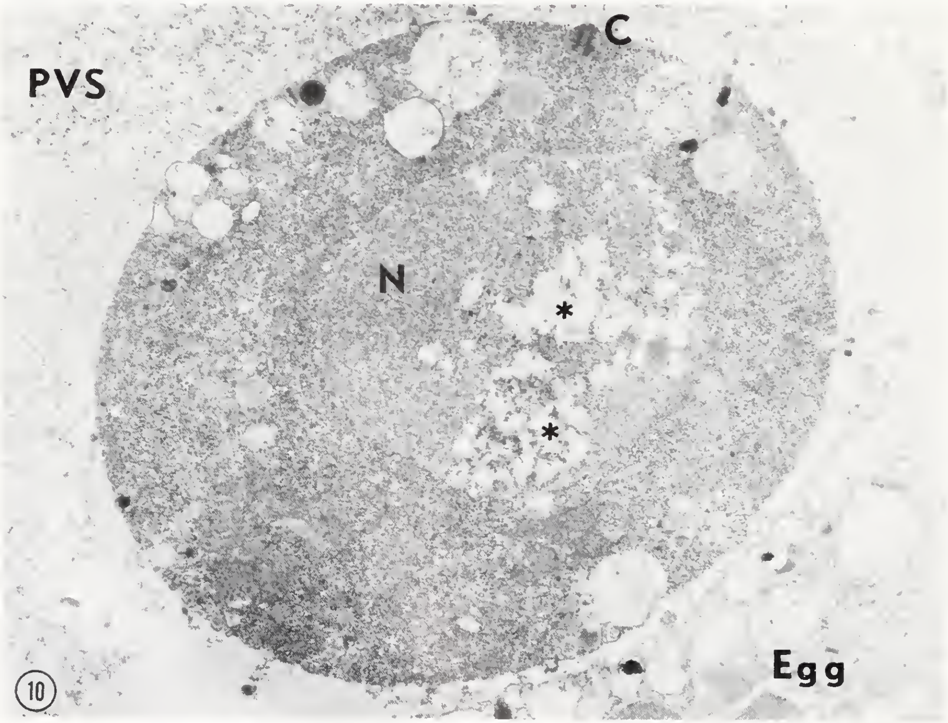
PVS
C
N
*
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Egg
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a substructure, were also present (Fig. 2). Small cisternae of endoplasmic reticulum, mostly of the smooth variety, as well as mitochondra were distributed throughout the cytoplasm. Golgi complexes were not prominent.

### *Germinal vesicle breakdown*

Breakdown of the germinal vesicle in oocytes matured naturally or with exogeneous 1-MA was morphologically similar; the observations provided below are taken from studies where maturation was initiated by exogenous 1-MA. Furthermore, the application of 1-MA to immature oocytes allowed for a precise timing of meiotic maturation. Hence, the times referred to herein are based on counts of eggs where greater than 50% demonstrated a given stage of development ($N > 100$) and where the moment of addition of 1-MA was time-zero.

One of the earliest signs of germinal vesicle breakdown was the modification of the periphery of the germinal vesicle; *i.e.*, its surface became convoluted by 15 minutes after the addition of 1-MA, and this was followed by the disruption of the nuclear envelope (Figs. 3, 4). The nuclear envelope vesicutated, such that numerous vesicles were found along the interface of cytoplasm and the nucleoplasm (Fig. 3). Concomitantly, the nucleolus assumed a highly irregular profile and dispersed (Fig. 4). Continued meiotic maturation led to a considerable reduction in the volume formerly occupied by the germinal vesicle (Figs. 5–7). At 30 minutes after the addition of 1-MA the nuclear envelope and much of the nucleolus had disappeared. By 40 minutes after the addition of 1-MA the condensing chromosomes were apparent as "clear" areas rather than the usual opaque structures obtained in fixed preparations of other cell types (Fig. 6). Evidently, the preparative methods employed in this study removed portions of the chromosomes.

Within 60 minutes following the application of 1-MA, the chromosomes were observed associated with the forming meiotic spindle which was usually located in the central portion of the egg (Figs. 6, 7). Relative to the size of the egg the meiotic apparatus was small, measuring about 10 $\mu$m in length. It lacked prominent asters and appeared "barrel-shaped" when sectioned longitudinally. The spindle migrated to the animal pole of the egg and underwent its meiotic divisions (Figs. 8, 9).

The second polar body of a fertilized starfish egg is shown in Figure 10, 120 minutes after addition of 1-MA. The chromosomes taken into the second polar

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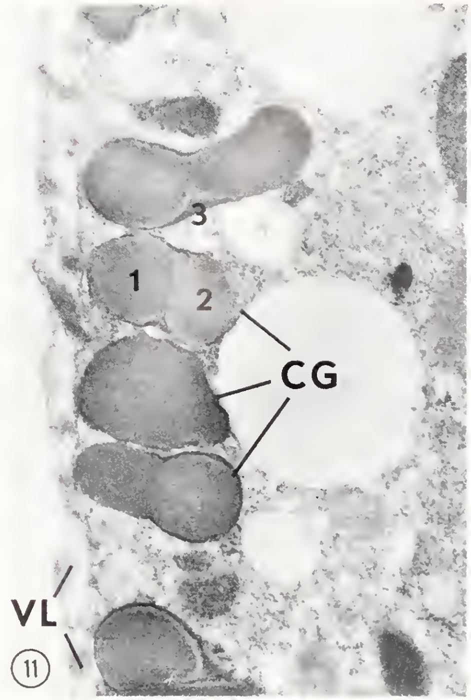
FIGURES 4–6. *Asterias* oocytes in successive stages of meiotic maturation. Eggs fixed at 15 (Fig. 4), 30 (Fig. 5), and 40 minutes (Fig. 6) after the addition of 1-MA. ×1150. Figure 4 depicts the development of plications along the periphery of the germinal vesicle (arrows), the disruption of the nucleolus (Nu), and the development of a "clearing" (C) of organelles along the periphery of the germinal vesicle. This is followed by the disappearance of the nuclear envelope (Fig. 5) and the condensation of the meiotic chromosomes (Ch), which are the lightly stained structures shown in the center of the egg in Figure 6. The structures depicted by arrows in Figure 5 are remnants of the nucleolus.

FIGURES 7 AND 8. Condensed chromosomes (arrows) organized on the meiotic spindle (MS) which is formed in the center of the egg (Fig. 7) and then moves to the cortex (Fig. 8). Specimens prepared at 60 and 70 minutes after addition of 1-MA, respectively.

FIGURE 9. *Asterias* egg having completed the formation of the first polar body (1PB); specimen fertilized after germinal vesicle breakdown. The chromosomes remaining within the egg are shown at the arrow, prior to their organization on the second meiotic spindle. ×1150.

FIGURE 10. Second polar body of a fertilized *Asterias* egg located within the perivitelline space (PVS) and containing a nucleus (N) and centriole (C). The areas within the nucleus indicated (*) represent chromatin which is dissolved by the preparative methods employed. × 36,000.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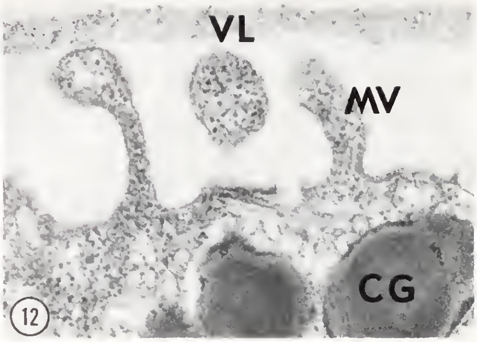
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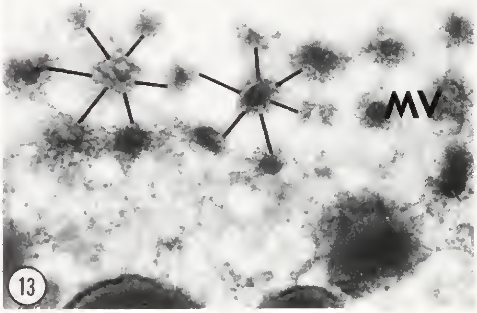
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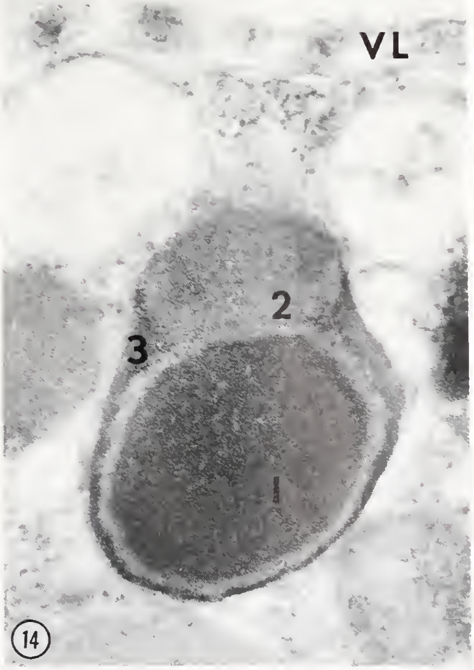
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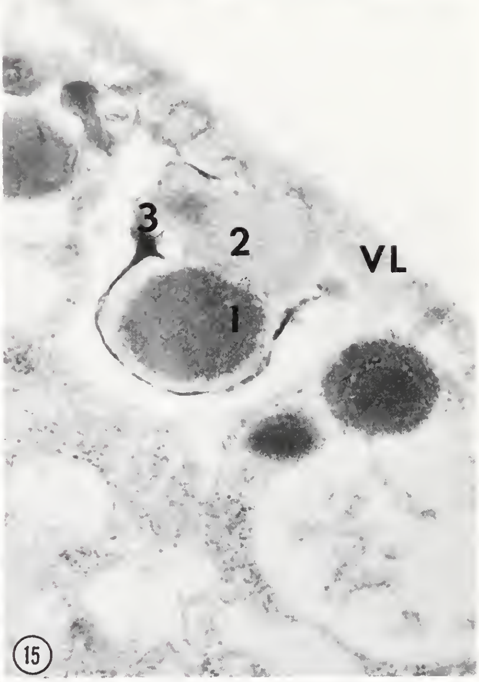
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body comprised a miniature nucleus. In addition to mitochondria and some vesicular structures, at least one centriole was observed within the second polar body.

### *Cortical granule reaction*

The cortex of immature and maturing (from both 1-MA treated specimens and spontaneously ovulating females) oocytes appeared morphologically similar to one another. The plasma membrane was projected into numerous microvilli that were arranged in a hexagonal pattern and were covered by a prominent vitelline layer, composed of a filamentous material (Figs. 11–13). Although in some specimens the vitelline layer was separated slightly from the surface of the egg and only covered the tips of the microvilli (Fig. 12), it was morphologically similar to those that surrounded the microvilli (Figs. 11, 13). The separation of the vitelline layer was a random occurrence, seemingly unrelated to 1-MA treatment; its basis was not established. A monolayer of ellipsoid cortical granules was located within the cortex (Figs. 11, 12). The long axis of the granules was positioned at a right angle to the surface of the egg and measured 2 to 2.5 $\mu$m in length (Fig. 11). Structurally the granules usually contained three components that were resolved at high magnification (Fig. 14). The first was a spheroid mass of dense material, having a fine-textured appearance, which was positioned in the distal and/or proximal portions of the granule. Second, was a fine granular material of lesser electron opacity that often surrounded the first and filled much of the remainder of the granule. The third component was dense, relatively sparse in comparison to the other two components, and usually confined to the lateral aspect of the granule. In addition to the cortical granules some vesicular elements and mitochondria were present in the cortex of maturing and immature oocytes (Figs. 11, 14).

Insemination initiated the cortical granule reaction which was morphologically the same in immature and maturing eggs and comparable to that previously described for sea urchins (Anderson, 1968; Millonig, 1969). Consequently, only micrographs of the cortical granule reaction in maturing eggs are presented. Because of the three morphologically distinct components of the starfish cortical granule, their fate and relation to one another with respect to the formation of the fertilization membrane and organization within the perivitelline space could be followed. A dehiscing cortical granule is shown in Figure 15. Soon after fusion of the cortical granule membrane with the plasma membrane all three components of the cortical

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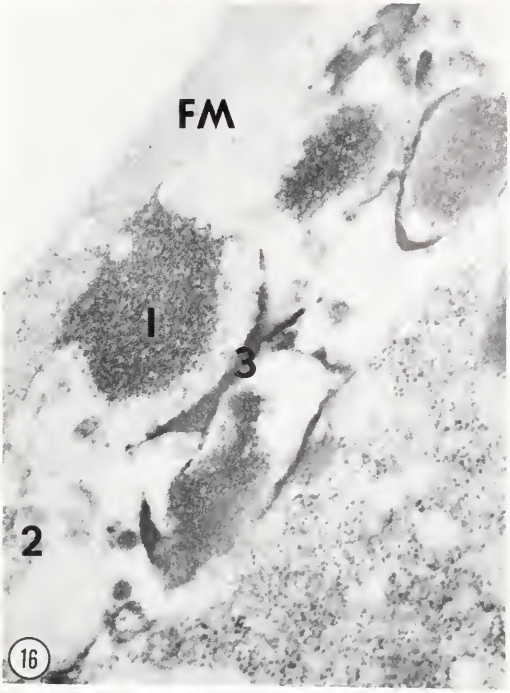
FIGURE 11. Cortex of an immature *Asterias* oocyte containing cortical granules (CG). The plasma membrane is covered by a vitelline layer (VL). Three structural components (1, 2, and 3; see text for explanation) may be seen in some cortical granules. × 27,000.

FIGURE 12. Portion of the cortex of an unfertilized maturing oocyte in which the vitelline layer (VL) has separated from the plasma membrane. Under these conditions the vitelline layer shows no changes in internal structure. MV, microvilli; CG, cortical granules. ×30,000.

FIGURE 13. Tangential section of the surface of a maturing *Asterias* egg demonstrating the organization of microvilli (MV). The filamentous material eminating from the microvilli represents a part of the vitelline layer. ×49,000.

FIGURE 14. Cortical granule of a maturing *Asterias* oocyte containing three structural components (1, 2, and 3; see text). A portion of the vitelline layer (VL) is depicted. ×33,000.

FIGURE 15. Dehiscing cortical granule of an inseminated, maturing oocyte. Initially, the more electron translucent component (2) is closely associated with the vitelline layer (VL) which is separating from the surface of the egg. Other components, which are still confined to the cup-like structure of the dehiscing cortical granule (1 and 3), are beginning to disperse. ×52,000.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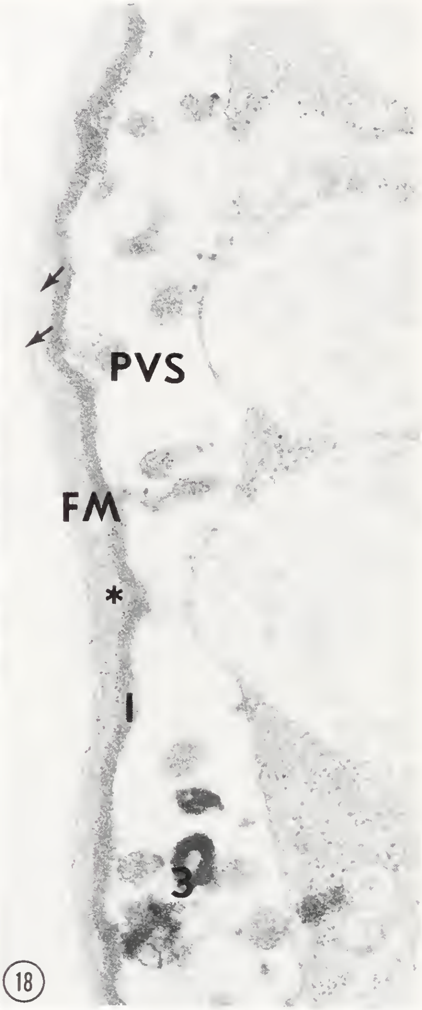
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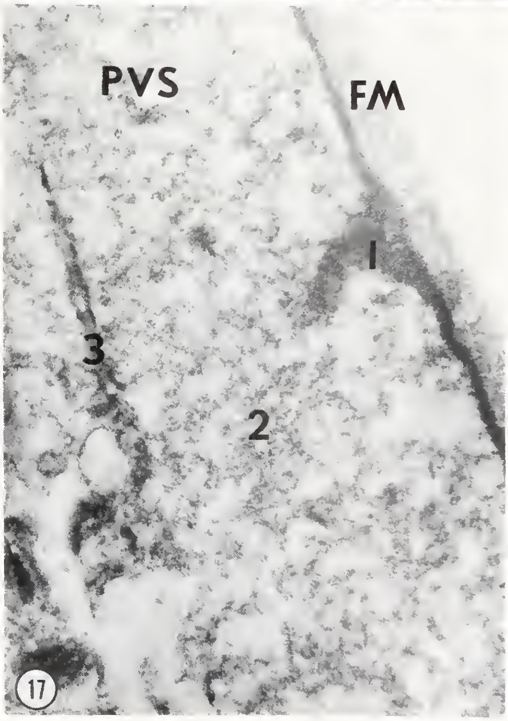
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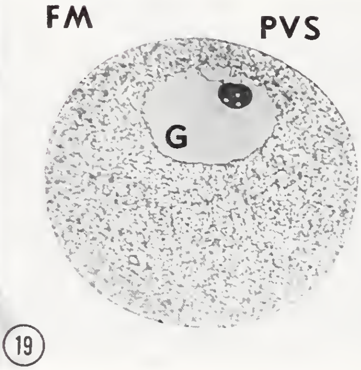
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granule appeared to swell. Initially the second component became associated with the vitelline layer. Later, however, much of this material appeared to disperse and fill the perivitelline space (Figs. 16, 17). The first component became associated with the vitelline layer; this material eventually coated the entire inner margin of the developing fertilization membrane and was seen as a dense layer (Figs. 16, 17). The fate of the third component was unclear; it appeared to form plate-like structures that were distributed throughout the perivitelline space (Figs. 16, 17).

Following the release of the cortical granule contents a well-defined fertilization membrane was formed in both immature and maturing ova (Fig. 18). Morphologically, it consisted of: (1) an outer laminated region apparently derived from the vitelline layer itself, with a possible contribution from the second component of the cortical granule, and (2) an electron opaque region along the innermost portion of the fertilization membrane consisting of material derived from the first component of the cortical granule (Fig. 18). The perivitelline space of both inseminated immature and maturing eggs was relatively large and measured up to 12 μm in width (Fig. 19); it was filled primarily with an electron translucent substance in which were found some dense structures apparently derived from the cortical granules.

## DISCUSSION

The observations of this study demonstrate: (1) morphological alterations and their chronology in *Asterias* eggs, induced by 1-MA, leading to the development of the second polar body, (2) the structure of the *Asterias* egg cortex and cortical granule reaction, and (3) that germinal vesicle-containing oocytes of *Asterias* are capable of undergoing a cortical granule reaction morphologically similar to that of eggs having undergone germinal vesicle breakdown.

### *Germinal vesicle breakdown and meiotic maturation*

The germinal vesicle of *Asterias* oocytes is morphologically comparable to those observed in eggs of other organisms (Kessel, 1968; Millonig *et al.*, 1968; Longo and Anderson, 1970). That 1-MA had an effect on meiotic maturation was first indicated by the undulation of the nuclear envelope of the germinal vesicle and a disruption of the nucleolus. These changes are characteristic of germinal vesicle breakdown as

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FIGURE 16. Cortex of a maturing oocyte, in which the contents of the cortical granules fill the perivitelline space. The cortical granule component designated 2 is dispersed within the perivitelline space and may have become integrated into the developing fertilization membrane (FM). Component 1 is seen as an electron dense aggregate closely associated with the inner margin of the developing fertilization membrane. Electron dense component 3 is distributed within the perivitelline space. ×49,000.

FIGURE 17. Perivitelline space (PVS) of a fertilized, mature oocyte in which one of the structural components (1) of the cortical granules has lined the inner surface of the developing fertilization membrane. The material distributed throughout the perivitelline space may be derived from component 2. The dense material (3) may be derived from the third component of the cortical granules. ×44,000.

FIGURE 18. Structural organization of the fertilization membrane (FM) of an inseminated, immature oocyte. The fertilization membrane has collapsed and, consequently, is located in close proximity to the egg surface. The outermost aspect of the fertilization membrane (*) consists of laminated regions (arrows). The innermost aspect of the fertilization membrane is lined by electron dense material derived from component 1 of the cortical granules. The dense material located within the perivitelline space (PVS) may be derived from component 3 of the cortical granules. ×28,000.

FIGURE 19. Immature ooctye 2 hours after insemination possessing a fertilization membrane (FM) and prominent perivitelline space (PVS). G, germinal vesicle. ×1300.

observed in oocytes of other organisms where meiotic maturation is induced by other means (Merchant and Chang, 1971; Calarco *et al.*, 1972; *cf.* Longo, 1973; Sorenson, 1973). The significance of the tortuous outline developed by germinal vesicles induced to break down has not been established. Similar distortions in nuclear structure observed in other cells may be due to fluxes of materials into and out of the nucleus (Monroy, 1965). Changes in the germinal vesicle of starfish oocytes induced by 1-MA are believed to be brought about by the production of maturation promotion factor which is also responsible for subsequent maturation events (Kishimoto *et al.*, 1981).

With the exception of the development of asters, formation of the meiotic apparatus in *Asterias* is similar to that described for *Spisula* and *Tubifex* (Longo and Anderson, 1969, 1970; Shimizu, 1981a, b). The meiotic spindle was formed in the central portion of the oocyte and then moved to the cortex. The meiotic spindle of *Asterias* was structurally similar to that observed in mouse eggs in that it lacked well-developed asters and was barrel-shaped (Szollosi *et al.*, 1972). Due to the relatively large size of oocytes we were unable to verify the appearance and number of centrioles in the meiotic spindles of *Asterias*; however, the presence of at least one centriole in the second polar body indicates that these organelles are probably an integral part of the meiotic apparatus. Thus, the situation differs from that observed in mammals. Characteristically, the meiotic spindle of mammalian oocytes lacks centrioles (Szollosi, 1972; Szollosi *et al.*, 1972).

### *Cortical granule reaction*

There has existed in the literature a question as to whether or not immature starfish oocytes are capable of a cortical granule reaction and the formation of a fertilization membrane (*cf.* Masui and Clarke, 1979). It has been generally believed that germinal vesicle breakdown was necessary before the starfish egg was capable of a cortical reaction (Hirai *et al.*, 1971; Hirai, 1976). However, immature eggs incubated in calcium-free sea water were able to inseminate and undergo a cortical granule reaction (Cayer *et al.*, 1975; Schuetz, 1975). The results presented herein support and amplify these observations at the ultrastructural level of observation and indicate that the cortical granule reaction in *Asterias* ova, with or without germinal vesicles, is structurally similar.

The cortical granule reaction in *Asterias* is morphologically similar to that described by Holland (1980) for *Patiria miniata.* Because of structurally recognizable cortical granule components, their fate and relation to development of the fertilization membrane can be traced. The present study shows that the dense component of the cortical granules coats the inner margin of the vitelline layer and becomes an integral part of the fertilization membrane. A similar process has also been described for sea urchins and *Patiria* (Anderson, 1968; *cf.* Ito, 1969; Inoué and Hardy, 1971; Holland, 1980).

Investigators working with the eggs of different organisms have shown that cortical granule components become a part of the vitelline layer and their interaction is related to characteristics the fertilization membrane acquires with its development, *e.g.*, hardening (Endo, 1961; Wolpert and Mercer, 1961; Bryan, 1970; Grey *et al.*, 1974; Chandler and Heuser, 1980; *cf.* Shapiro and Eddy, 1980; Schuel *et al.*, 1982). A similar interaction may also exist in *Asterias*. That the vitelline layer of *Asterias* showed no change in structure when separated from the surface of eggs not having undergone a cortical reaction suggests that cortical granule material is necessary for the progressive structuralization of the fertilization membrane.

Despite the release of the entire population of cortical granules and evidence from other echinoderms demonstrating that components of the hyaline layer are derived from cortical granules (Kane, 1970; Stephens and Kane, 1970; *cf.* Schuel, 1978), a well-defined hyaline layer was not obvious in fertilized eggs of *Asterias.* Although some of the cortical granule material is incorporated into the fertilization membrane, the fate of the remainder is in question. Some material is seen within the perivitelline space. However, it is much too sparse to form a prominent layer as found in many sea urchins. One reason for the absence of a layer may be due to the relatively larger perivitelline space, characteristic of fertilized *Asterias* eggs. The cortical granule contents may fill this space, resulting in a relatively diffuse distribution. Holland (1980, 1981) has questioned the presence of a hyaline layer in starfish as found in echinoids. As indicated by Hall and Vacquier (1982), participation by the hyaline layer does not appear to be greatly relevant to echinoderm morphogenesis, as this structure is seemingly found in only echinoids and ophiuroids (*cf.* also Holland, 1981). In starfish, the interaction of blastomeres alone without the aid of an extracellular layer seems to be sufficient for blastula formation (Dan-Sohkawa, 1976; Dan-Sohkawa and Fujisawa, 1980).

## ACKNOWLEDGMENTS

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# DISTRIBUTION AND ECOLOGY OF MYSIDS IN CAPE COD BAY, MASSACHUSETTS

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## ABSTRACT

Seven species of mysids (*Neomysis americana, Erythrops erythropthalma, Mysis stenolepis, Mysis mixta, Heteromysis formosa, Praunus flexuosus,* and *Meterythrops robusta*) were collected from Cape Cod Bay, Massachusetts. The general ecology of the first four species is described in terms of several classificatory schemes proposed for worldwide mysid distributions.

Organismal relationships to geographic, seasonal, bathymetric, bottom water temperature, and sediment characteristics are examined. Four species occurred year-round with the following seasonal peaks in abundance: *N. americana* (February, April, December), *E. erythropthalma* (January, March, December), *M. mixta* (March and July), *M. stenolepis* (January and August). Based on bathymetric and sedimentary relationships the species tend to occur in pairs. *Neomysis americana* and *M. stenolepis* were primarily collected in shallow water (10–29 m) and from sand and clayey-silt. *Erythrops erythropthalma* and *M. mixta* occurred in deeper water (20–39 m) and on clayey-silt and silt. In addition to seasonal effects, evidence indicates that interactions among depth, bottom water temperature, and sediment type strongly influenced the spatial zonation of Cape Cod mysids. The distribution and ecology of the four mysids generally conformed to worldwide classification schemes.

## INTRODUCTION

The Cape Cod Bay, Massachusetts biotic census was conducted to provide data on species composition, abundance, diversity, and trophic groupings of marine benthic organisms in regard to biotic and abiotic factors, and to provide a base for systematic and ecologic investigations of the Cape Cod Bay ecosystem and for assessment of change brought about by human activities (Carriker, 1972). The present account focuses on the mysidaceans from the biotic census.

Since mysidaceans form a conspicuous component of macrozooplankton in freshwater and oceanic environments and can form an important resource in food web dynamics, they have been extensively studied (Gordan, 1957). Research along the northeast coast of North America reflects this worldwide interest (Verrill *et al.*, 1873; Rathbun, 1905; Bigelow and Sears, 1939; Bousfield, 1956, 1961; Brunel, 1960; Wigley, 1963; Haefner, 1968). Although mysids are commonly considered to be planktonic, studies with a variety of bottom collectors have shown that some species are benthic or spend some portion of their life on the bottom (Clutter, 1967; Murano, 1970a, b). Based on $3 \times 10^6$ specimens collected from 1953 to 1969 from the continental shelf and slope between Canada and southern Florida, bathymetry, bottom

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sediment, and generation type were recognized as important features influencing mysid distribution (Wigley and Burns, 1971). In a comprehensive review Muachline (1980) proposed several classifications to describe worldwide mysid distributions based on these features. This research examines whether mysidaceans collected by the Cape Cod Bay biotic census were responding to the same features underlying Mauchline's (1980) classifications for worldwide distributions.

Cape Cod Bay is described elsewhere (Young and Rhoads, 1971). It encompasses 1600 km$^2$, is circular, and opens northward to Massachusetts Bay (Figure 1). Mean tidal range at Plymouth, Massachusetts is 2.9 m. Average annual extremes of surface temperature (−0.1 and 19.9°C) and salinity (31.0 and 33.2‰) are similar to bottom temperature (−0.1 and 17.7°C) and bottom salinity (31.2 and 32.3‰). Bottom temperatures ranged from −1.5 to 23.5°C. Highest and lowest values of both hydrographic features are normally associated with surface waters. A summer thermocline appears in April and disappears in October. Reverse thermoclines may occur at 15 to 25 m during mid-winter when bottom water may be 1 to 2.5°C warmer than surface water. Sediments consist of a mixture of clayey-silt, silt, sand, and gravel. Sand and silt each comprise approximately 40–45% of the bay sediments, and gravel comprises the smallest component of sediment (Young and Rhoads, 1971).

## MATERIALS AND METHODS

The methods used to collect and process the samples are described in detail by Young *et al.* (1971). Since the goals of the study were to maximize the number of

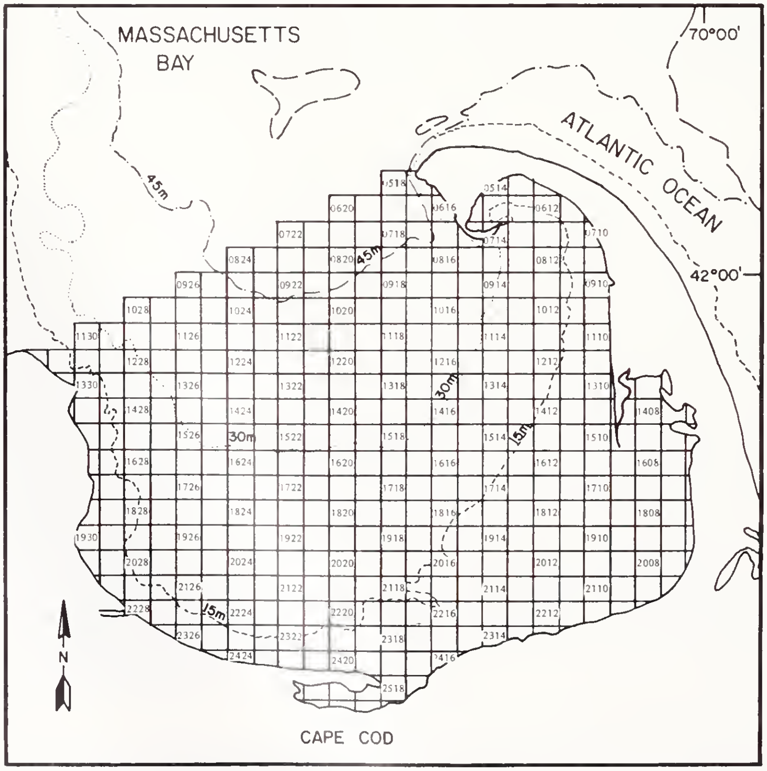
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FIGURE 1. Location of station quadrats, Cape Cod Bay, Massachusetts.

different locations sampled, no repetitive sampling over time was conducted. Cape Cod Bay was divided into one square mile quadrats (Figure 1). Sampling was conducted from 1966 to 1969, yielding samples from each month of the year. Although the sampling effort was evenly distributed over the bay, based on sediment distribution approximately 36.6 percent of the samples were taken from mud >4$\phi$ (where $\phi$ = median sediment particle size; 0.062 mm), 42.5 percent from very fine to coarse sand 1–4$\phi$ (0.062–0.50 mm), and 20.9 percent from coarse sand to gravel. Thus there is bias towards samples from coarser grained sediment. It should be emphasized that sampling was not synoptic and that seasonal patterns are based on a composite of samples collected over several years. Quadrats were sampled randomly over sediment type and depth range.

Quantitative samples were taken from the center and four corners of each complete quadrat by a Smith-McIntyre grab (0.1 $m^2$). Dredge hauls were obtained from three of the corners by towing to the center of each quadrat. The dredge types included an epibenthic sled, a modified commercial clam dredge, and a naturalist dredge.

Quantitative samples were washed immediately by elutriation with sea water into 1.0 and 0.5 mm screens, and dredge hauls were washed through the former. The washed residue on each screen was placed for 5–10 minutes in a 0.15% solution of propylene phenoxytol in sea water. Specimens were preserved in a 10% solution of formalin in sea water for 48–72 hours, rinsed with tap water for several minutes, and transferred to 85% ethyl alcohol for final storage. Preserved samples of mysids were sorted according to species, sex, and life stage and counted under microscopes.

Four hundred and sixty grab samples and 260 dredge hauls were collected. At the center of each quadrat, surface and bottom temperature and salinity were measured. Sediment cores for analysis of particle size were taken from each Smith-McIntyre sample and frozen until analyzed. A total of 320 sediment samples were analyzed. Textural analysis was done by dry sieving the sand fractions through an Udden-Wentworth sieve series on a RoTap shaker following initial dispersion with sodium metaphosphate. The silt and clay fractions were determined by pipette analysis. For purposes of this presentation gravel is defined as > −1.0$\phi$, sand −1.0 to 4.0$\phi$, silt 4.0 to 5.0$\phi$, and clayey-silt >5.0$\phi$.

The number, sex, and life stage (adult, immature, ovigerous, larvigerous) of individuals per each species of mysid were tabulated. The density (grab), relative abundance (dredge), and frequency (percent of occurrence) in grab and dredge samples were compared to environmental factors with correlation coefficients (R). Density was transformed by $\log_e (N + 1)$ prior to correlation. Analysis of covariance was performed on monthly density counts using biomedical computer programs from the University of California, Los Angeles. The program produced an analysis of variance for adjusted group means and a *t*-test matrix for adjusted group means. This procedure was used because of unequal data sets and because it tests whether the means of the dependent variable are significantly different among groups and whether the difference is due to differences in the independent variable among the groups (Snedecor and Cochran, 1967; Sokal and Rohlf, 1969).

## RESULTS

### *General occurrence*

*Neomysis americana* (Smith) was collected most frequently followed in descending frequency by *Erythrops erythropthalma* (Goes), *Mysis mixta* Lillgeborg, and *Mysis stenolepis* Smith. *Neomysis americana* occurred throughout the bay except the north central portion whereas *E. erythropthalma* and *M. mixta* occurred everywhere except the southern and southeastern portion. *Mysis stenolepis* occurred mainly in the southern half of the bay with a few occurrences in the northern half. Several specimens of *Heteromysis formosa* (Smith) and *Praunus flexuosus* (Müller) and a damaged specimen questionably assigned to *Meterythrops robusta* (Smith) were also collected.

### *Seasonal distribution*

*Neomysis americana* was collected every month with abundance peaks in February, April, and December (Table I). Based on Analysis of Variance (ANOVA, F = 2.48, D.F. 66,360) the effect of month of collection was statistically significant ($\alpha = 0.01$). Examination of the $t$-test matrix for adjusted group means of grab samples indicated that catches from February, April, and December were significantly different ($\alpha = 0.05$) from those in other months. Patterns based on dredge data showed high relative abundance during the same three months.

The overall sex ratio of adults was 0.51 males to 1 female (grab) and 0.82 males to 1 female (dredge). Dredge hauls yielded ovigerous stages in October and larvigerous stages from April through October.

*Erythrops erythropthalma* was collected every month, and number collected peaked in March and December (Table I). Based on ANOVA (F = 1.6, D.F. 66, 360), the effect of month was statistically significant ($\alpha = 0.01$). The $t$-test matrix for grab data indicated that January and December were significantly different ($\alpha = 0.05$). Trends depicted by dredge data indicated relatively large numbers January through April (Table I).

Density of *E. erythropthalma* decreased throughout spring and summer (May–August). The overall sex ratio of adults was 0.32 males to 1 female (grab) and 0.53 males to 1 female (dredge). Dominance in sex ratio of *E. erythropthalma* changed more frequently throughout the year than did that of *N. americana*. June and November were the only months when immature forms were not collected by dredge. Dredge collections produced ovigerous stages in May and July (Table I). Larvigerous stages were collected in January, May–September, and December.

*Mysis mixta* was collected every month but October (Table I). Based on ANOVA (F = 2.7, D.F. 66,360), the effect of month of sampling was significant ($\alpha = 0.01$). the $t$-test matrix for grab data indicated that the majority of monthly samples were significantly different ($\alpha = 0.05$) from one another. Dredge data indicated a peak in March followed by a rapid decline and gradual increase through July (Table I). Immature forms occurred from April to July. Ovigerous forms were collected in January and December, and larvigerous stages were taken from January to April. The sex ratio of adults was 0.02 males to 1 female (grab) and 0.07 males to 1 female (dredge).

Results for *M. stenolepis* are primarily based on dredge data as less than 2% were collected quantitatively (Table I). *M. stenolepis* was collected every month, with peak abundances in January and August. Ovigerous forms were only collected in January, whereas larvigerous forms were collected in January, March, and April. The sex ratio of adults was 0.1 males to 1 female.

### *Relationship to bathymetry*

Most *N. americana* in Cape Cod Bay were collected in shallow to intermediate depths (Table II). There was a rapid decline in number caught at depths greater than 40 m. The highest density occurred at 30–39 m and the highest relative abundance

TABLE I

*Monthly distribution of* Neomysis americana, Erythrops erythropthalma, Mysis mixta *and* Mysis stenolepis *by sex and stage in Cape Cod Bay.*

| | Grab (No./m$^2$) | | | | | Dredge (No./haul) | | | | | | | Grab (No./m$^2$) | | | | | Dredge (No./haul) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M | F | Imm | Lar | Total | M | F | Imm | Ovig | Lar | Total | Grand total | M | F | Imm | Lar | Total | M | F | Imm | Ovig | Lar | Total | Grand total |
| | *Neomysis americana* | | | | | | | | | | | | *Erythrops erythropthalma* | | | | | | | | | | | |
| Jan | 5 | 33 | 5 | 0 | 43 | 39 | 131 | 34 | 0 | 0 | 204 | 247 | 8 | 22 | 14 | 0 | 44 | 41 | 90 | 23 | 0 | 2 | 156 | 200 |
| Feb | 122 | 90 | 20 | 0 | 232 | 168 | 72 | 15 | 0 | 0 | 255 | 487 | 13 | 9 | 2 | 0 | 24 | 52 | 71 | 17 | 0 | 0 | 140 | 164 |
| Mar | 4 | 13 | 2 | 0 | 19 | 32 | 63 | 8 | 0 | 0 | 103 | 122 | 11 | 27 | 16 | 0 | 54 | 182 | 317 | 116 | 0 | 0 | 615 | 669 |
| Apr | 38 | 120 | 8 | 0 | 166 | 380 | 142 | 61 | 0 | 2 | 585 | 751 | 2 | 23 | 8 | 0 | 33 | 71 | 49 | 16 | 0 | 0 | 136 | 169 |
| May | 2 | 6 | 0 | 1 | 9 | 76 | 86 | 7 | 0 | 24 | 193 | 202 | 1 | 6 | 0 | 0 | 7 | 21 | 58 | 9 | 5 | 2 | 95 | 102 |
| Jun | 0 | 12 | 91 | 0 | 103 | 23 | 22 | 4 | 0 | 4 | 53 | 156 | 1 | 6 | 1 | 1 | 9 | 22 | 38 | 0 | 0 | 0 | 60 | 69 |
| Jul | 0 | 2 | 2 | 1 | 5 | 5 | 8 | 2 | 0 | 1 | 16 | 21 | 2 | 7 | 0 | 0 | 9 | 2 | 11 | 3 | 1 | 1 | 18 | 27 |
| Aug | 20 | 21 | 35 | 0 | 76 | 51 | 68 | 23 | 0 | 7 | 149 | 225 | 0 | 4 | 6 | 0 | 10 | 26 | 49 | 4 | 0 | 3 | 82 | 92 |
| Sep | 4 | 4 | 2 | 0 | 10 | 10 | 14 | 3 | 0 | 2 | 29 | 39 | 8 | 18 | 7 | 1 | 34 | 11 | 17 | 6 | 0 | 0 | 34 | 68 |
| Oct | 3 | 10 | 21 | 0 | 34 | 57 | 64 | 104 | 12 | 9 | 246 | 280 | 12 | 17 | 5 | 0 | 34 | 6 | 3 | 1 | 0 | 0 | 10 | 44 |
| Nov | 2 | 23 | 15 | 0 | 40 | 2 | 1 | 8 | 0 | 0 | 11 | 51 | 1 | 2 | 0 | 0 | 3 | 1 | 3 | 0 | 0 | 0 | 4 | 7 |
| Dec | 28 | 51 | 32 | 0 | 111 | 48 | 348 | 35 | 0 | 0 | 431 | 542 | 2 | 47 | 15 | 0 | 64 | 4 | 100 | 14 | 0 | 4 | 122 | 186 |
| Total | 228 | 385 | 233 | 2 | 848 | 891 | 1019 | 304 | 12 | 49 | 2275 | 3123 | 61 | 188 | 74 | 2 | 325 | 439 | 806 | 209 | 6 | 12 | 1472 | 1797 |
| | *Mysis mixta* | | | | | | | | | | | | *Mysis stenolepis* | | | | | | | | | | | |
| Jan | 0 | 3 | 0 | 0 | 3 | 20 | 22 | 0 | 7 | 5 | 54 | 57 | 0 | 0 | 0 | 0 | 0 | 12 | 10 | 0 | 7 | 1 | 30 | 30 |
| Feb | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 1 | 5 | 5 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 |
| Mar | 0 | 1 | 0 | 0 | 1 | 8 | 134 | 0 | 0 | 48 | 190 | 191 | 0 | 1 | 0 | 0 | 1 | 0 | 8 | 0 | 0 | 7 | 15 | 16 |
| Apr | 0 | 0 | 0 | 0 | 0 | 0 | 29 | 2 | 0 | 3 | 34 | 34 | 0 | 0 | 0 | 0 | 0 | 1 | 11 | 0 | 0 | 5 | 17 | 17 |
| May | 0 | 19 | 5 | 0 | 24 | 0 | 63 | 38 | 0 | 0 | 101 | 125 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 2 | 2 |
| Jun | 0 | 22 | 13 | 0 | 35 | 0 | 52 | 65 | 0 | 0 | 117 | 152 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Jul | 0 | 7 | 0 | 0 | 7 | 0 | 149 | 110 | 0 | 0 | 259 | 266 | 0 | 0 | 0 | 0 | 0 | 0 | 14 | 0 | 0 | 0 | 14 | 14 |
| Aug | 0 | 18 | 0 | 0 | 18 | 4 | 136 | 0 | 0 | 0 | 140 | 158 | 0 | 0 | 0 | 0 | 0 | 0 | 26 | 0 | 0 | 0 | 26 | 26 |
| Sep | 0 | 9 | 0 | 0 | 9 | 0 | 16 | 0 | 0 | 0 | 16 | 25 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 0 | 0 | 0 | 10 | 10 |
| Oct | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 0 | 0 | 0 | 20 | 20 |
| Nov | 0 | 0 | 0 | 0 | 0 | 5 | 4 | 0 | 0 | 0 | 9 | 9 | 0 | 0 | 0 | 0 | 0 | 5 | 9 | 0 | 0 | 0 | 14 | 14 |
| Dec | 2 | 5 | 0 | 0 | 7 | 18 | 17 | 0 | 1 | 0 | 36 | 43 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 2 | 2 |
| Total | 2 | 84 | 18 | 0 | 104 | 55 | 626 | 215 | 8 | 57 | 961 | 1065 | 0 | 2 | 0 | 0 | 2 | 20 | 111 | 0 | 7 | 13 | 151 | 153 |

M = male; F = female; Imm = immature; Ovig = ovigerous; Lar = larvigerous.
No ovigerous individuals were collected by the grab method.

occurred at 20–29 m. Peak density of immature stages occurred at 10–19 m and peak relative abundance of immature, ovigerous, and larvigerous stages was at 0.19 m (Table II). The frequency and relative abundance of *N. americana* (dredge) decreased significantly ($\alpha = 0.01$) with depth ($R = -0.56$, $R = -0.35$) as did the frequency in grab samples ($R = -0.29$, $\alpha = 0.05$).

Maximum numbers of *E. erythropthalma* were collected in intermediate depths in Cape Cod Bay (Table II). There was a marked increase in numbers at depths

TABLE II

*Relationship to bathymetry (m) of common mysids by sex and stage in Cape Cod Bay.*

| Species Bathymetric Range (m) | Grab (No./m$^2$) | | | | | Dredge (No./haul) | | | | | | Grand total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M | F | Imm | Lar | Total | M | F | Imm | Ovig | Lar | Total | |
| *N. americana* | | | | | | | | | | | | |
| 0–9 | 30 | 41 | 53 | 1 | 125 | 121 | 166 | 58 | 1 | 11 | 357 | 482 |
| 10–19 | 17 | 60 | 127 | 1 | 205 | 127 | 265 | 136 | 11 | 34 | 573 | 778 |
| 20–29 | 37 | 103 | 25 | 0 | 165 | 445 | 507 | 96 | 0 | 4 | 1052 | 1217 |
| 30–39 | 142 | 176 | 27 | 0 | 345 | 186 | 66 | 11 | 0 | 0 | 263 | 608 |
| 40–49 | 2 | 5 | 1 | 0 | 8 | 11 | 15 | 3 | 0 | 0 | 29 | 37 |
| 50–59 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 |
| Total | 228 | 385 | 233 | 2 | 848 | 891 | 1019 | 304 | 12 | 49 | 2275 | 3123 |
| *E. erythropthalma* | | | | | | | | | | | | |
| 0–9 | 0 | 2 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 0 | 1 | 3 |
| 10–19 | 2 | 6 | 1 | 0 | 9 | 10 | 43 | 2 | 4 | 2 | 61 | 70 |
| 20–29 | 30 | 59 | 21 | 1 | 111 | 162 | 240 | 88 | 0 | 0 | 490 | 601 |
| 30–39 | 7 | 48 | 14 | 1 | 70 | 211 | 354 | 91 | 1 | 9 | 666 | 736 |
| 40–49 | 17 | 58 | 37 | 0 | 112 | 45 | 134 | 18 | 0 | 0 | 197 | 309 |
| 50–59 | 5 | 15 | 1 | 0 | 21 | 10 | 35 | 10 | 1 | 1 | 57 | 78 |
| Total | 61 | 188 | 74 | 2 | 325 | 439 | 806 | 209 | 6 | 12 | 1472 | 1797 |
| *M. mixta* | | | | | | | | | | | | |
| 0–9 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 0 | 0 | 3 | 3 |
| 10–19 | 0 | 13 | 14 | 0 | 27 | 0 | 47 | 6 | 0 | 8 | 61 | 88 |
| 20–29 | 0 | 15 | 1 | 0 | 16 | 16 | 98 | 7 | 2 | 14 | 137 | 153 |
| 30–39 | 0 | 25 | 2 | 0 | 27 | 12 | 342 | 142 | 0 | 4 | 500 | 527 |
| 40–49 | 2 | 27 | 1 | 0 | 30 | 16 | 105 | 59 | 3 | 31 | 214 | 244 |
| 50–59 | 0 | 4 | 0 | 0 | 4 | 11 | 32 | 0 | 3 | 0 | 46 | 50 |
| Total | 2 | 84 | 18 | 0 | 104 | 55 | 626 | 215 | 8 | 57 | 961 | 1065 |
| *M. stenolepis* | | | | | | | | | | | | |
| 0–9 | 0 | 0 | 0 | 0 | 0 | 3 | 19 | 0 | 0 | 1 | 23 | 23 |
| 10–19 | 0 | 2 | 0 | 0 | 2 | 5 | 53 | 0 | 0 | 5 | 63 | 65 |
| 20–29 | 0 | 0 | 0 | 0 | 0 | 9 | 31 | 0 | 4 | 5 | 49 | 49 |
| 30–39 | 0 | 0 | 0 | 0 | 0 | 1 | 6 | 0 | 1 | 1 | 9 | 9 |
| 40–49 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 0 | 2 | 1 | 7 | 7 |
| 50–59 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total | 0 | 2 | 0 | 0 | 2 | 20 | 111 | 0 | 7 | 13 | 151 | 153 |

M = male; F = female; Imm = immature; Ovig = ovigerous; Lar = larvigerous.
No ovigerous individuals were collected by the grab method.

greater than 20 m. The highest density occurred between 20 and 29 m. Immature forms reflected the same density distribution patterns as adults. Ovigerous and larvigerous stages (dredge) were collected from 10 to 59 m with a peak for the latter in the 30–39 m range. The frequency (grab) of this species increased significantly ($\alpha = 0.01$) with depth (R = 0.48).

Maximum numbers of *M. mixta* were found at middle depths (Table II). Density of immature stages was highest at 30–39 m. Ovigerous and larvigerous stages also tended to occupy middle depths (Table II). The frequency of *M. mixta* in grab (R = 0.28) and dredge (R = 0.27) samples increased significantly ($\alpha = 0.05$) with depth.

The depth range of *Mysis stenolepis* resembled that of *N. americana* more than that of the other two common mysids (Table II). The highest relative abundance occurred at 10–19 m and declined rapidly at depths greater than 30 m. Larvigerous stages occurred from 0 to 49 m and ovigerous stages occurred from 20 to 49 m. Frequency and relative abundance decreased (R = −0.33, R = −0.28) significantly ($\alpha = 0.05$) with depth.

## *Relationship to bottom water temperature*

Most *N. americana* were caught in bottom waters at temperatures between −1.5 and 8.1°C (Table III). Numbers declined above 8.1°C. Density of immature stages was highest between 6.0 and 8.1°C and relative abundance of immature stages was highest at 8.2 to 12.0°C. Ovigerous stages were collected at 8.2 to 12.0°C, and larvigerous stages (dredge) were sampled at temperatures of 3.3 to 23.5°C. The density and relative abundance of *N. americana* decreased (R = −0.47, R = −0.46) significantly ($\alpha = 0.01$) with increasing temperature.

Most *E. erythropthalma* occurred from −1.5 to 8.1°C (Table III) with a marked decline above 8.1°C. Immature, ovigerous, and larvigerous stages were found at the same temperature range as adults. Density (R = −0.65), relative abundance (R = −0.54), and frequency (grab R = −0.52, dredge R = −0.76) of *E. erythropthalma* decreased significantly ($\alpha = 0.01$) with increasing temperature.

Maximum densities of *M. mixta* occurred at temperatures of 3.3 to 8.1°C, whereas maximum relative abundance occurred from −1.5 to 8.1°C (Table III). Immature stages from both types of collecting gear were most abundant from 3.3 to 8.1°C. In contrast, ovigerous and larvigerous stages (dredge) were relatively more abundant between −1.5 and 5.9°C. The frequency of *M. mixta* in grab (R = −0.38) and dredge samples (R = −0.67) decreased significantly ($\alpha = 0.05$, $\alpha = 0.01$) with increasing temperature.

Relative abundance of *M. stenolepis* was generally high throughout a range of −1.5 to 23.5°C (Table III). This was the most eurythermal species of the common Cape Cod Bay mysids. Immature stages were more abundant in warmer temperatures (6.0–23.5°C), whereas ovigerous and larvigerous stages were more abundant below 6.0°C. The frequency and relative abundance of *M. stenolepis* decreased with increasing temperature, but the relationships were not statistically significant.

## *Relationship to sediment type*

Maximum density of *N. americana* occurred in clayey-silt with relatively high numbers in sand and silt (Table IV). This species was also collected infrequently in gravel. Maximum relative abundance occurred in sand, followed in decreasing order by clayey-silt and silt, and gravel. Ovigerous and larvigerous stages were only collected in sand. The frequency (dredge) of *N. americana* decreased with increasing

TABLE III

*Bottom water temperature distribution of common mysids by sex and stage in Cape Cod Bay.*

| Species Temperature Range (°C) | Grab (No./$m^2$) | | | | | Dredge (No./haul) | | | | | | Grand total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M | F | Imm | Lar | Total | M | F | Imm | Ovig | Lar | Total | |
| *N. americana* | | | | | | | | | | | | |
| −1.5–3.2 | 130 | 137 | 29 | 0 | 296 | 234 | 281 | 58 | 0 | 0 | 573 | 869 |
| 3.3–5.9 | 40 | 122 | 8 | 0 | 170 | 439 | 198 | 70 | 0 | 4 | 711 | 881 |
| 6.0–8.1 | 35 | 71 | 131 | 1 | 238 | 108 | 381 | 43 | 0 | 26 | 558 | 796 |
| 8.2–12.0 | 2 | 25 | 19 | 0 | 46 | 56 | 66 | 104 | 12 | 8 | 246 | 292 |
| 12.1–23.5 | 21 | 30 | 46 | 1 | 98 | 54 | 93 | 29 | 0 | 11 | 187 | 285 |
| Total | 228 | 385 | 233 | 2 | 848 | 891 | 1019 | 304 | 12 | 49 | 2275 | 3123 |
| *E. erythropthalma* | | | | | | | | | | | | |
| −1.5–3.2 | 29 | 51 | 30 | 0 | 110 | 264 | 451 | 147 | 0 | 2 | 864 | 974 |
| 3.3–5.9 | 16 | 69 | 20 | 1 | 106 | 123 | 184 | 45 | 2 | 1 | 355 | 461 |
| 6.0–8.1 | 4 | 50 | 21 | 1 | 76 | 49 | 166 | 16 | 4 | 9 | 244 | 320 |
| 8.2–12.0 | 12 | 16 | 2 | 0 | 30 | 3 | 5 | 1 | 0 | 0 | 9 | 39 |
| 12.1–23.5 | 0 | 2 | 1 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 3 |
| Total | 61 | 188 | 74 | 2 | 325 | 439 | 806 | 209 | 6 | 12 | 1472 | 1797 |
| *M. mixta* | | | | | | | | | | | | |
| −1.5–3.2 | 0 | 4 | 0 | 0 | 4 | 21 | 143 | 0 | 5 | 48 | 217 | 221 |
| 3.3–5.9 | 2 | 47 | 4 | 0 | 53 | 14 | 177 | 98 | 2 | 9 | 300 | 353 |
| 6.0–8.1 | 0 | 33 | 14 | 0 | 47 | 15 | 300 | 116 | 1 | 0 | 432 | 479 |
| 8.2–12.0 | 0 | 0 | 0 | 0 | 0 | 5 | 6 | 1 | 0 | 0 | 12 | 12 |
| 12.1–23.5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total | 2 | 84 | 18 | 0 | 104 | 55 | 626 | 215 | 8 | 57 | 961 | 1065 |
| *M. stenolepis* | | | | | | | | | | | | |
| −1.5–3.2 | 0 | 1 | 0 | 0 | 1 | 14 | 14 | 0 | 7 | 4 | 39 | 40 |
| 3.3–5.9 | 0 | 0 | 0 | 0 | 0 | 1 | 15 | 0 | 0 | 9 | 25 | 25 |
| 6.0–8.1 | 0 | 1 | 0 | 0 | 1 | 4 | 34 | 0 | 0 | 0 | 38 | 39 |
| 8.2–12.0 | 0 | 0 | 0 | 0 | 0 | 1 | 20 | 0 | 0 | 0 | 21 | 21 |
| 12.1–23.5 | 0 | 0 | 0 | 0 | 0 | 0 | 28 | 0 | 0 | 0 | 28 | 28 |
| Total | 0 | 2 | 0 | 0 | 2 | 20 | 111 | 0 | 7 | 13 | 151 | 153 |

M = male; F = female; Imm = immature; Ovig = ovigerous; Lar = larvigerous.
No ovigerous individuals were collected by the grab method.

median sediment size ($\phi$) (R = −0.64, $\alpha = 0.01$), increased with percent sand (R = 0.53, $\alpha = 0.01$), and decreased with percent clayey-silt (R = −0.51, $\alpha = 0.01$). Relative abundance also declined with percent silt (R = −0.37) and the frequency (grab) decreased with percent clayey-silt (R = −0.57, $\alpha = 0.01$).

Maximum density of *E. erythropthalma* was in clayey-silt and silt, with immature stages most abundant in clayey-silt (Table IV). Maximum relative abundance occurred in silt, clayey-silt, and sand. Ovigerous and larvigerous stages were collected throughout a range of sand to clayey-silt. The frequency of *E. erythropthalma* in grab (R = 0.78) and dredge (R = 0.72) samples, density (R = 0.59), and relative abundance (R = 0.66) increased significantly ($\alpha = 0.01$) with decreasing $\phi$. The fre-

TABLE IV

*Sediment distribution of common mysids by sex and stage in Cape Cod Bay.*

| Species Sediment Type | Grab (No./$m^2$) | | | | | Dredge (No./haul) | | | | | | Grand total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M | F | Imm | Lar | Total | M | F | Imm | Ovig | Lar | Total | |
| *N. americana* | | | | | | | | | | | | |
| gravel | 0 | 7 | 1 | 0 | 8 | 12 | 11 | 9 | 0 | 0 | 32 | 40 |
| sand | 46 | 99 | 181 | 2 | 328 | 527 | 426 | 215 | 12 | 49 | 1229 | 1557 |
| silt | 16 | 63 | 22 | 0 | 101 | 208 | 516 | 66 | 0 | 0 | 790 | 891 |
| clayey-silt | 166 | 216 | 29 | 0 | 411 | 144 | 66 | 14 | 0 | 0 | 224 | 635 |
| Total | 228 | 385 | 233 | 2 | 848 | 891 | 1019 | 304 | 12 | 49 | 2275 | 3123 |
| *E. erythropthalma* | | | | | | | | | | | | |
| gravel | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| sand | 2 | 8 | 1 | 0 | 11 | 82 | 106 | 21 | 4 | 2 | 215 | 226 |
| silt | 20 | 68 | 23 | 0 | 111 | 240 | 437 | 133 | 1 | 5 | 816 | 927 |
| clayey-silt | 39 | 112 | 50 | 2 | 203 | 117 | 263 | 55 | 1 | 5 | 441 | 644 |
| Total | 61 | 188 | 74 | 2 | 325 | 439 | 806 | 209 | 6 | 12 | 1472 | 1797 |
| *M. mixta* | | | | | | | | | | | | |
| gravel | 0 | 5 | 2 | 0 | 7 | 0 | 0 | 0 | 0 | 0 | 0 | 7 |
| sand | 0 | 18 | 12 | 0 | 30 | 0 | 74 | 15 | 0 | 11 | 100 | 130 |
| silt | 0 | 28 | 4 | 0 | 32 | 48 | 378 | 200 | 5 | 32 | 663 | 695 |
| clayey-silt | 2 | 33 | 0 | 0 | 35 | 7 | 174 | 0 | 3 | 14 | 198 | 233 |
| Total | 2 | 84 | 18 | 0 | 104 | 55 | 626 | 215 | 8 | 57 | 961 | 1065 |
| *M. stenolepis* | | | | | | | | | | | | |
| gravel | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| sand | 0 | 1 | 0 | 0 | 1 | 9 | 87 | 0 | 0 | 10 | 106 | 107 |
| silt | 0 | 1 | 0 | 0 | 1 | 8 | 11 | 0 | 5 | 3 | 27 | 28 |
| clayey-silt | 0 | 0 | 0 | 0 | 0 | 3 | 13 | 0 | 2 | 0 | 18 | 18 |
| Total | 0 | 2 | 0 | 0 | 2 | 20 | 111 | 0 | 7 | 13 | 151 | 153 |

M = male; F = female; Imm = immature; Ovig = ovigerous; Lar = larvigerous.
No ovigerous individuals were collected by the grab method.

quency and density increased significantly ($\alpha = 0.01$) with percent silt (R = 0.70, R = 0.52) and percent clayey-silt (R = 0.67, R = 0.58), while relative abundance increased significantly ($\alpha = 0.05$) with percent silt (R = 0.34). In contrast, the frequency of *E. erythropthalma* in grab (R = −0.50) and dredge (R = −0.53) samples, density (R = −0.50), and relative abundance (R = −0.36) decreased significantly ($\alpha = 0.01$, $\alpha = 0.01$, $\alpha = 0.01$, $\alpha = 0.05$) with percent sand.

*Mysis mixta* occurred at greatest density in sand through clayey-silt (Table IV), with immature stages primarily in sand. Maximum relative abundance occurred in silt followed by clayey-silt and sand. Larvigerous and ovigerous stages were found throughout a sand to clayey-silt range. The frequency of *M. mixta* (dredge) increased significantly with increasing $\phi$ (R = 0.68, $\alpha = 0.01$), percent silt (R = 0.34, $\alpha = 0.05$), and percent clayey-silt (R = 0.44, $\alpha = 0.05$) and decreased with percent sand (R = −0.51, $\alpha = 0.01$). The frequency of this species (grab) decreased significantly ($\alpha = 0.05$) with percent sand (R = −0.28) and increased with percent silt (R = 0.32) and percent clayey-silt (R = 0.45, $\alpha = 0.01$). The density decreased significantly ($\alpha = 0.01$) with percent sand (R = −0.46).

Most *M. stenolepis* were caught in sand (Table IV). Larvigerous stages were relatively more abundant in sand, but ovigerous stages were collected in silt and clayey-silt. The relative abundance of *M. stenolepis* significantly increased ($\alpha = 0.05$) with percent sand (R = 0.33) and decreased with percent silt (R = −0.42).

## DISCUSSION

### *Collecting gear*

The grab sample data presented here provide some of the first quantitative estimates of densities of life history stages of mysids in relation to seasonal and environmental factors for the northeast United States. However, there is some collecting bias between the grab and dredges. Dredge hauls frequently collected more life history stages, particularly ovigerous and larvigerous forms, than grab samples (Table I). Moreover, grab samples underestimated the frequency and numbers of *M. stenolepis* (Table I). Accordingly one's perception of mysid distribution patterns can be significantly affected by type of collecting gear used (Mauchline, 1980).

### *Geographic distribution*

*Erythrops erythropthalma* and *M. mixta* are considered amphi-Atlantic species. In contrast, *M. stenolepis* and *N. americana* are considered warm temperate to tropical water species (Wigley and Burns, 1971). Mauchline (1980) stated that species living south of 60°N, including *E. erythropthalma* and *M. mixta*, may intrude into the Arctic Ocean regularly or sporadically. According to him *M. stenolepis* and *N. americana* belonged to a fauna confined to the western Atlantic between 60°N and 40°N. *Mysis mixta* was also considered amphi-Atlantic by Mauchline (1980) but also characteristic of coastal areas. Occurrence in New England waters is well documented for species collected in this study (Fish, 1925; Whiteley, 1948; Wigley, 1964).

### *Seasonal distribution*

Wigley and Burns (1971) found ovigerous and larval stages of *N. americana* from March to October with the largest numbers in March through June and August through October along the northeastern U. S. continental shelf and slope. Immature stages were particularly numerous in August and December. The situation in Cape Cod Bay differed in that large pulses of adults occurred in February, April, and December, ovigerous stages occurred only in October, and larval stages occurred from April to October with a May peak (Table I). Hopkins (1965) reported three major spawning peaks of *N. americana* (April–May, June, August) in Delaware Bay. He encountered a few ovigerous stages as late as January and February. Williams (1972) reported the greatest abundance of *N. americana* from November to May or June in North Carolina estuaries. He showed that ovigerous or larvigerous stages occurred in every month but November.

Mauchline (1980) proposed several major types of mysid reproduction and succession of generations. His classification included species with 0.5, <1, 1, 2, 3, and ≥3 generations per year. *Neomysis americana* may not fit easily into Mauchline's (1980) classification scheme. There is evidence to indicate that *N. americana* produces two generations a year on Georges Bank (Wigley and Burns, 1971) and in Cape Cod Bay (Table I), three in Delaware shallow waters (Hopkins, 1965), and perhaps three or more generations in North Carolina estuaries (Williams, 1972). If this pattern is accurate, it suggests a latitudinal shift of reproduction for *N. americana.*

According to Wigley and Burns (1971), ovigerous stages of *E. erythropthalma* occurred only in August and larvigerous stages in August and September along the Atlantic coast. This contrasts with our findings in Cape Cod Bay of ovigerous stages in May and July and larvigerous stages in January, May–September, and December (Table I). These findings tend to confirm the tentative conclusion of a lengthy spawning period proposed by Wigley and Burns (1971). *Erythrops erythropthalma* probably produces two generations per year and falls within Mauchline's (1980) classification.

Wigley and Burns (1971) concluded that *M. mixta* had two definite age groups in both spring and fall. Immature stages were common in May and October. No ovigerous specimens were present in their collections. The only indication of spawning season was the presence of small (5.3–6.3 mm) individuals in May, suggesting a late winter or early spring spawning. Within Cape Cod Bay, adult, peaks occurred in later winter and summer with a July peak for immature stages (Table I). Tattersall (1951) recorded many occurrences of adults in August and September but made no references to larvigerous and ovigerous stages. Records of ovigerous stages in January and larvigerous stages in January through April (March peak) (Table I) confirm the late winter/early spring spawning period proposed by Wigley and Burns (1971). The grossly unbalanced sex ratio reported for *M. mixta* from broad ranging samples on the continental shelf was also recorded in the more restricted confines of Cape Cod Bay. Different habitat preferences for males and females, different environmental conditions for reproduction and larval development, or short-lived life cycle for males may explain this pattern.

Although data for *M. stenolepis* are sparse, there is a suggestion of peaks for adults in winter (January) and summer (August) and for larvigerous stages in late winter/early spring (Table I). Ovigerous stages were collected only in January. This view agrees with earlier versions provided by Smith (1879) and Tattersall (1951). According to Mauchline's scheme, *M. stenolepis* and probably *M. mixta* would belong to species producing one generation per year.

### *Relation to bathymetry*

Mauchline (1980) proposed a bathymetric classification of mysids that included recognition of the ecological significance of salinity (freshwater and brackish). Exclusive of brackish and freshwater species, he recognized a spectrum ranging from littoral, to shallow shelf, to eurybenthic shelf, to bathypelagic.

Wigley and Burns (1971) established five depth categories from which mysids were most frequently caught. *Neomysis americana*, E. erythropthalma, and *M. mixta* were listed as eurybathic shelf species (range 1–421 m), and *M. stenolepis* was cited as a shore species (intertidal). Within Cape Cod Bay there was evidence of spatial partitioning in terms of bathymetric stratum. *Neomysis americana* and *M. stenolepis* were characteristic of shallow and intermediate depths, while *E. erythropthalma* and *M. mixta* were characteristic of intermediate to greater depths (Table II). The associations of *N. americana* and *M. stenolepis* with shallow water and of *E. erythropthalma* with deeper water were reported previously (Segerstrale, 1945; Tattersall, 1951, 1954; Bousfield, 1956; Wigley, 1964; Wigley and Burns, 1971). In contrast, Hulburt (1957) found more *N. americana* at greater depths in Delaware Bay. Their low abundance in shallow water may have been due in part to the presence of caridean shrimp (*Crangon septemspinosa* (Say), *Palaemontes vulgaris* (Say), *P. pugio* (Holthuis)) which are very abundant in shallow waters of Delaware Bay (Price, 1962).

The segregation of Cape Cod Bay species pairs by depth is indicative of zonation, which reduces competition for space. Zonation of nearshore mysids (0–17 m) was described from a sand bottom on the open coast of California (Clutter, 1967). He concluded that zonation probably developed in response to the availability of food imposed by nearshore circulation. This relationship cannot be ignored in Cape Cod Bay; a case for multivariate environmental interaction is discussed later. In terms of Mauchline's (1980) bathymetric classification *N. americana* and *M. stenolepis* in Cape Cod Bay would fall within the littoral to shallow shelf habitat and *E. erythropthalma* and *M. mixta* would fit the shallow shelf to eurybenthic shelf habitat.

### *Relationship to bottom water temperature*

*Neomysis americana* is considered to be eurythermic, found at bottom water temperatures from 0 to 25°C (Wigley and Burns, 1971). Within Cape Cod Bay *N. americana* occurred throughout a similar temperature range, but their maximum distribution was between −1.5 and 8.1°C (Table III). Specimens from Delaware Bay were most abundant at lower temperatures (Hulburt, 1957). *Erythrops erythropthalma* showed a bottom water temperature distribution similar to that of *N. americana*, but its abundance peaked in even lower temperatures (−1.5 to 3.2°C) (Table III). *Mysis stenolepis* occurred throughout the local temperature range, but *M. mixta* was only collected below 12.0°C (Table III). The peak of the latter species was 6.0–8.1°C. The former species, together with *N. americana*, occurred in appreciable numbers above 12.0°C. The local temperature occurrence of the two species of *Mysis* was consistent with their shallow and deeper water habits.

Bottom water temperature changes seasonally. However, there was evidence for interaction between temperatures and depth on abundance and frequency of mysids. Maximum abundance of *N. americana*, *E. erythropthalma*, and *M. stenolepis* occurred in a temperature range of −1.5 to 8.1°C, which coincided with high seasonal numbers recorded for January through March. A similar relationship can be seen for *M. mixta* with maximum abundance in 6.0–8.1°C which coincided with high seasonal numbers recorded for May and July. Seasonal effects are evident in these relationships.

However, the relationship between bottom water temperature and the deeper water mysid pair (*E. erythropthalma* and *M. mixta*) was further complicated by depth-temperature interaction. A marked summer thermocline was reported in Cape Cod Bay from mid-April until Mid-October (Young *et al.*, 1971). The annual temperature at 20 to 26 m ranged from −1.5 to 10°C. The lowest extent of the thermocline defined by the 5°C isotherm intersected the sea floor at approximately 26 m. Even though mysid distribution is influenced by seasonal effects of bottom water temperature, the latter is influenced by bathymetry. It might be expected that the shallow water pair of mysids are more responsive to seasonal water temperatures, whereas the deeper water pair are more affected by depth-bottom temperature relationships. Emberton (1981) showed that selected taxa of subtidal meiofauna in Cape Cod Bay were significantly influenced by season-depth interactions. Season was more important in shallow water, whereas there was a time lag at greater depths in terms of meiofauna density.

### *Relationship to sediment type*

Mauchline (1980) cited many cases of sediment preference for hyperbenthic mysids. Wigley and Burns (1971) summarized the sediment distribution of mysids as follows: *N. americana* and *E. erythropthalma* on sand, *M. stenolepis* on sand and *Zostera*, and *M. mixta* on a variety of sediments. However, the present study showed that silt and clayey-silt played an important role in the distribution of Cape Cod Bay mysids (Table IV). Williams (1972) cited evidence that *N. americana* in North Carolina estuaries commonly occurred over sediments of clay and silt-sized particles. Young and Rhoads (1971) collected quantitative samples in Cape Cod Bay and reported *N. americana* from sand and clayey-silt.

In this study, *N. americana* had a wide sediment range occurring in gravel through clayey-silt (Table IV). Even though maximum abundance (dredge) was reported in sand, relatively high numbers were also recorded in silt and clayey-silt. Moreover, maximum density was recorded from clayey-silt. This broad sediment range, together with its eurythermic characterization and broad salinity range (Hulburt, 1957), is consistent with its occupancy of coastal areas and estuaries which normally display rapidly changing environmental conditions. The detrital load of estuaries and the feeding habit of *N. americana* are also involved in this association.

In view of the bias toward sand samples in the collection, maximum abundance and occurrence of *E. erythropthalma* in silt and clayey-silt indicates the importance of this sediment type. This relationship is supported by other findings (Young and Rhoads, 1971). Thus, the earlier view of the sediment distribution of *E. erythropthalma* as characteristically occurring in sand (Wigley and Burns, 1971) should be amended to include bottoms with significant amount of silt and clayey silt.

*Mysis mixta* had a broad sediment range comparable to *N. americana* but occurred most abundantly in Cape Cod Bay in silt and clayey-silt (Table IV). This distribution is generally consistent with an earlier view (Wigley and Burns, 1971). *Mysis stenolepis* peaked in sand in Cape Cod Bay, but this species may live in sediment containing as high as 18% silt or 12% clayey-silt. It appears that fine grain sediment can be considerably more important in the ecology of these four mysids than previously recognized. The relationships among fine sediment, particulate organics, microbiota, and mysid feeding habits deserve attention (Mauchline, 1980) because *M. stenolepis* may be able to digest cellulose (Wainwright and Mann, 1982).

There was evidence of significant relationships between mysid frequency, density, and relative abundance and sediment type. Sediment decreases in modal size and increases in total clay and carbon contents with depth in Cape Cod Bay, and seston flux is 10 times greater at the deeper muddy stations than at the shallow sandy stations (Young *et al.*, 1971). Young *et al.* reported difficulty in determining which environmental factor was most important in separating zones of polychaetes in Cape Cod Bay because the isopleths of 10°C, 15–20 m, and 20% mud closely coincided. Their findings are consistent with the view of interactions among bottom water temperatures, depth, and sediment distribution. Accordingly, distributions of mysid species are probably influenced by these interactions. Since the same interactions are not as well defined along the northeastern part of the bay, seasonal-temperature factors may be more important here.

In summary, zonation of these mysids was related to depth-temperature-sediment interactions within a seasonal framework. These multifactorial environmental effects were expressed by a shallow water, silt-sand to sand pair of mysids (*N. americana* and *M. stenolepis*) and a deeper water, silt to clayey-silt pair (*E. erythropthalma* and *M. mixta*). Evidence for partitioning related to biotic factors was not included in this study, but evidence of considerable predation on mysids by finfish and competition through co-occurrences of mysids has been presented elsewhere (Wigley and Burns, 1971; Mauchline, 1980). In general, these mysids fall within Mauchline's (1980) bathymetric and reproductive classifications, with differences from these distributions associated with regional and seasonal conditions.

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# MEMBRANE-STABILIZING AND CALCIUM-BLOCKING AGENTS AFFECT *ARBACIA* SPERM MOTILITY

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## ABSTRACT

The speed and duration of progressive motility of *Arbacia* sperm cells depend on the calcium content of the suspension medium. Suspended in filtered sea water (FSW) the spermatozoa undergo a progressive decline in motility (after an initial burst of activity) and cease forward movement within 30–40 min. When sperm are diluted in chemically defined artificial sea water (MBL-ASW), motility rose to about 160% of the control rate in 30 min and then gradually returned to the initial control level where it persisted for at least 40 min more. Procaine, propranolol, ouabain, and quinidine, tested singly or in combination, affected sperm motility in both time- and concentration-dependent fashion.

Procaine at 10 and 100 $\mu M$/l in MBL-ASW caused more than a doubling in motility over the control rate, while in FSW both these concentrations were inhibitory. In FSW, quinidine had relatively little effect, while propranolol was slightly stimulatory at $10^{-6}\,M$ and inhibitory at 0.1 and $1.0 \times 10^{-3}\,M$. In combination, propranolol and quinidine can cause a sharp rise in motility. Ouabain increased motility dramatically in MBL-ASW suspensions. The effects of some of the drugs depend on the ability to displace calcium from binding sites in sperm cell membranes; ouabain appears to interface with Ca efflux.

## INTRODUCTION

Receptor activation and membrane lability play critical roles in the activity of many cell types. For example, induction of platelet aggregation by specific agonists is inhibited by substances classified as local anesthetics and antiarrhythmic agents; calcium antagonizes these inhibitory actions (Anderson *et al.*, 1981). Further, the effects of $Ca^{2+}$ in the medium on ciliary beat reversal in paramecium has been amply documented (Murakami and Eckert, 1972).

Similarly the movement of the mature spermatozoa of mammals and marine invertebrates is greatly influenced by interactions between sperm cell components and environmental factors. Responsiveness of sperm cell receptors to ligands, activators, and inhibitors, appears to vary with the condition of the sperm cell, its state of dilution (Gray, 1928; Rothschild, 1953), maturation (Babcock *et al.*, 1979), aging (Dunham *et al.*, 1982), capacitation, and even proximity to the ovum (Yanagimachi, 1970).

In the presence of some agents, other conditions being equal, the rate of sperm cell propulsion increases considerably. This implies that, under usual circumstances, not all of the sperm cells in a given sample are progressing at their maximal speed;

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Abbreviations: EDTA, ethylene diamine tetra acetate; EGTA, ethylene glycol bisaminoethyltetra acetate; FSW, filtered sea water; MBL-ASW, Marine Biological Laboratory formulated artificial sea water.

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that is, there seems to be a margin of safety which may permit the conservation of energy stores or otherwise enhance the union of physiologically uncompromised gametes. The difference between the optimum and the maximum swim speed capacity suggests the presence in the sperm cell of a regulatory mechanism modulated by control of calcium entry and transport through the cell as proposed here.

Procaine, added to sea water suspensions of *Arbacia* sperm, caused a rapid rise in their mean rate of forward motion followed by a sharp decline (Nelson, 1972). The local anesthetic apparently occupied binding sites in the plasma membrane, and having driven some of the bound calcium into the cell interior, then prevented its efflux.

The critical role of $Ca^{2+}$ in the modulation of sperm motility was further emphasized in studies with $ZnCl_2$, $MnCl_2$, and EDTA (Young and Nelson, 1974a) and $CaCl_2$, $LaCl_3$, and EGTA (Young and Nelson, 1974b). $Zn^{2+}$ and $Ca^{2+}$ had distinctly biphasic effects, while $Mn^{2+}$ and $La^{3+}$ as well as EDTA and EGTA were inhibitory or ineffective in the concentration ranges tested.

Cholinergic mediation appears to be involved in regulation of the entry of calcium into the sperm cell through specific ion channels (Nelson *et al.*, 1980). That is, calcium transport seems to depend on acetylcholine-induced conformational changes in a receptor channel complex that extends through the plasma membrane, similar to that proposed by Cohen and Changeux (1975) for cationic transport at myoneural junctions and electroplaques.

*Arbacia* sperm cells respond to nicotine, maximum stimulation occurring at $10^{-9}$ *M* and inhibition commencing at $10^{-6}$ *M*. The highly selective nicotinic receptor blocker, $\alpha$-bungarotoxin, completely inhibits all the cells in a suspension of *Arbacia* sperm at less than $10^{-6}$ *M*; microscopic examination showed that individual cells ceased moving at a concentration of less than 1 picomole/l (Nelson, 1976).

In mammalian sperm catecholamine sensitivity appears at the onset of the cytostructural and permeability changes coincident with capacitation (Bavister *et al.*, 1976; Cornett and Meizel, 1978), but neither epinephrine nor norepinephrine was observed to exert any appreciable change in the swim speed of *Arbacia* sperm that had not been exposed to capacitating conditions (unpublished observations).

Local anesthetics that block nerve conduction and have pronounced effects on muscle contraction raise the threshold for osmotic hemolysis of erythrocytes and interfere with platelet aggregation. The action of procaine on *Arbacia* spermatozoa attests to excitability as a physiological characteristic of the regulatory processes governing the movement of these cells.

This report extends studies on the effects of procaine on sea urchin sperm (Nelson, 1972) to include an examination of the action of the $\beta$-adrenergic blocking agent propranolol and the $\alpha$-blocker quinidine which when applied directly to cardiac muscle exerts an action similar to that of procaine. Propranolol is effective in combatting cardiac glycoside intoxication, and so the interactive effects of propranolol and ouabain on sperm motility were also examined.

## MATERIALS AND METHODS

Semen was collected daily from mature *Arbacia punctulata* induced to spawn by injection of 1 ml of 0.5 *M* KCl through the oral surface of the animal. The sea urchins were inverted over 30-ml beakers filled with either filtered sea water (FSW) or chemically defined Marine Biological Laboratory artificial sea water (MBL-ASW). (The dense semen streams settled rapidly and coherently to the bottom of the beaker without dispersing). This procedure permits the preparation of samples from the same sea urchin for suspension in either FSW or MBL-ASW, for use for an entire series of experimental runs. The supernatant fluid was decanted and the concentrated sperm cells were aspirated and transferred by means of disposable Pasteur pipettes into test tubes kept in an ice bath for the day's tests. As needed, sufficient concentrated sperm was diluted in 25 ml of FSW or MBL-ASW to yield an optical density reading between 0.500 and 0.700 in a Turner Model 350 Spectrophotometer ($\lambda = 480$ nm), equivalent to 7–10 × $10^6$ sperm/ml (Nelson, 1972).

For each experiment, different concentrations of the test reagents were quantitatively added by micropipet to 6 separate round cuvettes and the volume brought to 0.5 ml with FSW or MBL-ASW. The tests were initiated by addition of 2.0 ml of the sperm suspension diluted immediately prior to the start of each run. The "zero-time" reading was taken in the spectrophotometer after first mixing the cuvette contents by twice inverting the parafilm-covered tube. The cuvettes were then put into the six-place horizontal rotor of an I.E.C. clinical centrifuge and spun for 4 minutes at 120 × *g* (940 rpm); this has empirically been shown to align the spermatozoa with only minimal centrifugal sedimentation of non-motile cells (*ibid.*).

Orientation of the spermatozoa subjected to low centrifugal force permits reproducible measurement of changes in optical density of the suspensions as the cells swim past the light path. As the cells are stimulated, depressed, or unaffected by varying concentrations of a given combination of agents, optical density differences between the untreated controls and the treated suspensions are recorded from the spectrophotometer. The difference in O.D. between the zero time and 4-min centrifugal runs of the various specimens (after correction for displacement of formalin-killed cells, if any, is made) is determined. All the tubes in that series are normalized to the 4-min control reference point, as percent of control motility. Motility refers to progressive motion. (For full details, see Nelson, 1972.) All of the test reagents employed—procaine (free base); ouabain · $8H_2O$; DL-propranolol · HCl, and quinidine · $SO_4$—were of the purest grade available from Sigma Chemical Company. Artificial sea water (MBL-ASW) prepared in the Chemical Department of the Marine Biological Laboratory, Woods Hole, MA, contained (in m*M* per liter of deionized water): NaCl, 423; KCl, 9.0; $CaCl_2 \cdot 2H_2O$, 9.27; $MgCl_2$, 22.94; $MgSO_4$, 25.50; just prior to use 0.18 mg $NaHCO_3$/l was added. The inorganic salts were of analytical reagent grade, meeting ACS specifications.

All experiments were conducted at room temperature which ranged from 22.5° to 25°C during the course of the season.

## RESULTS

This group of test agents was selected because $Ca^{2+}$ has been implicated as a second messenger in cellular responses to their action. The swimming capacity of *Arbacia* sperm cells has long been known to deteriorate within 30–60 minutes after dilution in sea water. This so-called "dilution effect" starts with sharp increases in oxygen uptake and rate of movement which presumably rapidly deplete energy stores. Figure 1 illustrates the loss of motility of the FSW-diluted sperm cells, dropping to zero within 40 minutes. The abrupt rise in activity was not evident in these determinations since the first motility rating was not scored until five to six minutes after dilution. In the sperm samples suspended in MBL-ASW a protracted rise in the motility rate occurs that peaks at about 20 minutes and returns to the initial level for the duration of the experiment. When the sperm cells are suspended in a 90:10 mixture of MBL-ASW:FSW, the rate of increase and the maximum rate are both reduced and the sperm cell motility then gradually drops down to about half the speed in the MBL-ASW alone.

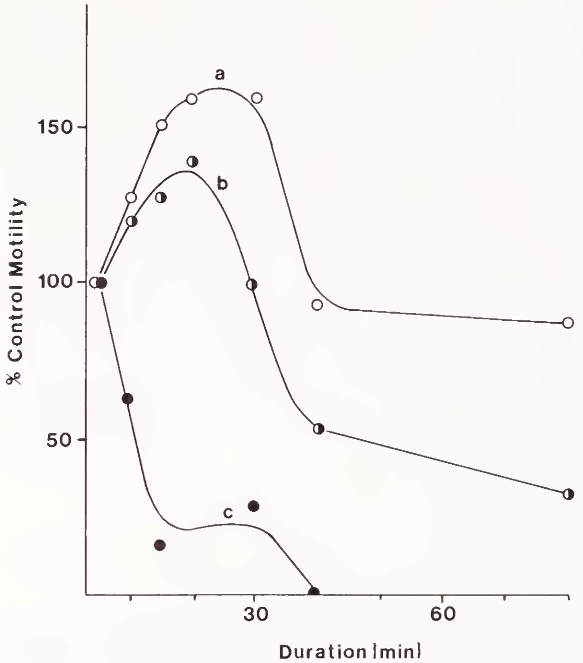

FIGURE 1. Dilution effects: dependence of rate and duration of *Arbacia* sperm swimming on calcium content of the suspension medium: a) open circles, MBL artifical sea water (MBL-ASW); b) half-circles, 90:10 mixture of MBL-ASW:FSW; c) closed circles, filtered sea water (FSW). Ordinate, relative speed of sperm cell progression (as percent of control) following dilution; abscissa, time elapsed after dilution. Sperm cells in MBL-ASW attain higher speeds and endure for longer periods than those in filtered sea water. Temp. 22.5°C.

In previous studies, the immediate effects of several concentrations of calcium and of procaine were examined. The present results with procaine indicate that both time dependence and concentration dependence of the response are modulated by the relative amounts of contaminants (presumably traces of heavy metals) in the medium. Figures 2a (FSW) and 2b (MBL-ASW) show close replication of the respective controls (no procaine) between the duration of sperm cell exposure to filtered sea water and the artificial sea water demonstrated in Fig. 1. In the presence of procaine, the sperm cells in FSW (Fig. 2a) generally undergo a fairly precipitous decline in motility, paralleling the control curve; $10^{-2}$ *M* procaine is predictably inhibitory from the start, the lower concentrations not differing significantly from the controls. With MBL-ASW as the suspending medium (Fig. 2b), even the $10^{-2}$ *M* procaine shows an initial, pronounced, increased acceleratory effect, the swimming speed rising to about 170% of the control rate in 15 minutes. The speed returns to the control levels by 20 minutes and then approaches a plateau while the controls continue their downward rate. Sperm cells suspended in millimolar procaine closely parallel the controls for the first twenty minutes but then decline much less abruptly. The spermatozoa in $10^{-5}$ and $10^{-4}$ *M* procaine, however, peak at nearly double the control speed in ten minutes and decrease gently to a level of forward motion 3–4 times greater than that of the untreated controls.

Procaine acts at the sperm cell surface; purportedly it affects cationic channels involved in calcium entry by displacing calcium from binding sites. Conversely ouabain, a specific inhibitor of $Na^+$, $K^+$-activated, $Mg^{2+}$-dependent adenosinetri-

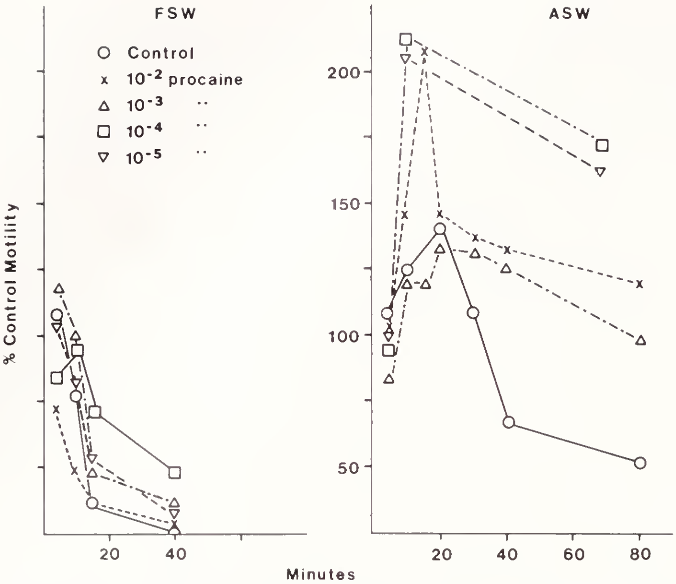

FIGURE 2. Effects of procaine on motility of *Arbacia* sperm suspended in a) FSW and b) MBL-ASW. Ordinate, percent of control motility rate; abscissa, time elapsed following dilution. Note that procaine-treated sperm suspended in MBL-ASW swim at a higher rate of speed for longer periods than do similarly treated sperm in FSW.

phosphatase, is considered to impede $Ca^{2+}$ efflux from cardiac muscle cells (Wood *et al.*, 1972) and may similarly affect sperm cells (*cf.* review, Nelson and McGrady, 1981). The maximum effect of a 10-min incubation in ouabain in filtered sea water occurs at about $10^{-6}$ *M*. This is shown in Figure 3 and confirms the previous report (Nelson, 1972). Increasing the incubation periods in MBL-ASW shifts the maximum response to the left; incubation in $10^{-9}$ *M* ouabain produces a peak in 30 minutes. This is again a dramatic (2.5 fold) increase over that of the initial rate of the FSW controls.

Ouabain toxicity in the mammalian heart cell may be counteracted by the beta-adrenergic-blocking agent propranolol (which in itself exhibits some of the Ca-perturbing properties of a local anesthetic). Propranolol and ouabain were therefore assayed singly and in combination; their interactive effects were tested on the progressive motility of sperm in filtered sea water. Lower concentrations of propranolol have little effect except for a 20% increase at $10^{-6}$ *M* (Fig. 4). However, at 0.1 m*M* a 40% decrease in motility occurs, while the inhibition increases to 80% at 1 m*M*. The peak effect of ouabain alone (at $10^{-6}$ *M*) was a 65% increase in the swimming rate over the controls. In the optimum concentration of ouabain ($10^{-6}$ *M*), increasing amounts of propranolol tend to lower the motility response curve by about 5–10%. Above the optimum concentration of both drugs ($10^{-6}$ *M* each), the ouabain did not significantly influence the response to propranolol. However, with ouabain set a concentration of $10^{-3}$ *M* throughout, the responses to varying amounts of propranolol are markedly altered. Both the prominent peak at $10^{-6}$ *M* and the profound depression at higher propranolol concentrations are eliminated.

Cinchona alkaloids reportedly exhibit digitalis-like properties. Therefore a further test of propranolol in drug-interactive effects on the sperm cell's ability to swim progressively is afforded in the experiments with quinidine. As in the preceding

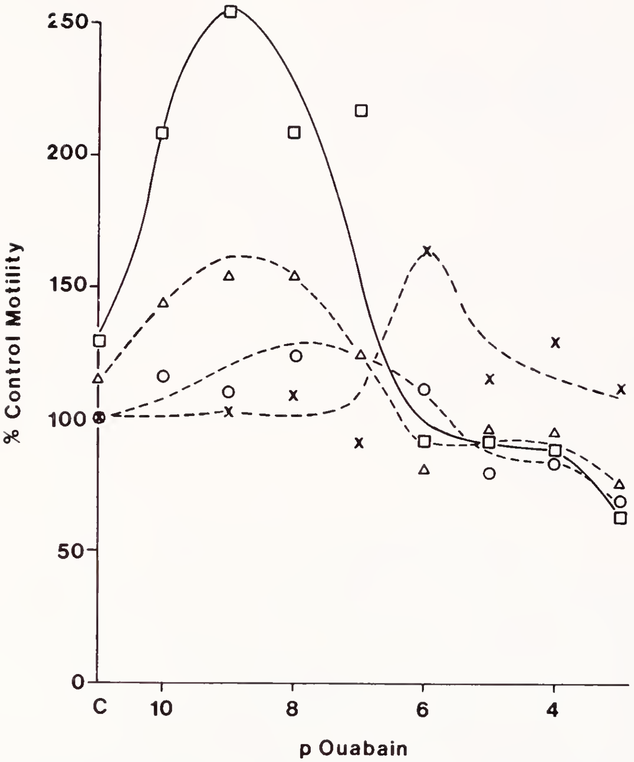

FIGURE 3. Time- and dose-dependent effects of ouabain on *Arbacia* sperm in FSW and MBL-ASW. X's, after 10 min exposure in FSW. Open symbols, in MBL-ASW: circles, 10 min; triangles, 20 min; squares, 30 min after dilution. Ordinate, relative motility in percent of control rate; abscissa, negative log of molar concentration of ouabain in the medium.

experiment (Fig. 4), the peak in $10^{-6}$ *M* propranolol is succeeded by a sharp motility decline with increasing drug concentration. Figure 5a shows that prolonging the incubation in propranolol has little added effect at the lower concentrations, but at micromolar amounts stimulation becomes evident and inhibition is somewhat ameliorated at concentrations of $10^{-5}$ *M* and higher.

Incubation in quinidine alone in FSW (shown in Fig. 5b) over a range of concentrations from $10^{-10}$ *M* to $10^{-4}$ *M* evokes a somewhat uneven but insignificant oscillation around the control rate of movement. The delayed effect on motility does not deviate strikingly from that of the delayed controls when the "dilution" effect is taken into account, *viz.*, a 60% to 80% decrease in progressive movement which is sustained over the entire concentration range after an additional ten minutes of incubation. When the two drugs are tested for interactive effects, the samples incubated in $10^{-3}$ *M* quinidine responded more vigorously than those in $10^{-5}$ *M*. In these experiments, the *Arbacia* sperm cells in seawater suspension were preincubated for five minutes, and, after their motility was rated, to each cuvette was added 0.2 ml FSW in the single treatment labeled "Q" or "P" or 0.2 ml of quinidine for the co-incubation, double-treatment series, labeled "P + Q" in the bar graph diagrams (Figs. 6a & 6b). After the additions, the cuvettes were again inverted 2 times to assure uniform redistribution. The sperm cells were then reoriented centrifugally, and readings were taken at the indicated intervals. Sperm cells exposed

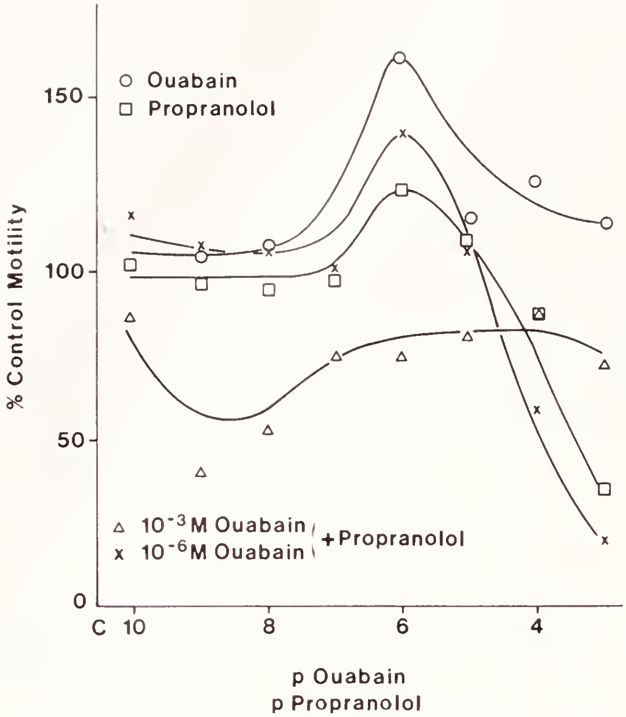

FIGURE 4. Interactive effects following 10-min incubation in the beta receptor blocker propranolol and the cardiac glycoside ouabain on *Arbacia* sperm motility in FSW. Note that the optimum concentration of each drug separately occurs at $10^{-6}$ *M*/l FSW; $10^{-6}$ *M* is also the optimum concentration for both drugs combined. Also note that in the presence of 1 m*M* ouabain, all concentrations of propranolol depressed motility. Ordinate, percent of control progressive motility rate; abscissa, concentrations of the drugs in negative log of molarity.

to $10^{-5}$ *M* quinidine after preincubation in $10^{-4}$ *M* or $10^{-6}$ *M* propranolol (Fig. 6a) show relatively little effect compared to those in $10^{-5}$ *M* quinidine alone.

In marked contrast sperm cells exposed to $10^{-3}$ *M* quinidine following their preincubation in $10^{-4}$ *M* and $10^{-6}$ *M* propranolol respectively, first responded with motility increases, ranging from 160% to 200% of the rates in quinidine alone (Fig. 6b). These bursts of activity were succeeded by precipitous declines to about 50% of the control and quinidine-alone rates on prolonged exposure of the sperm in both cases. In terms of initial reaction to addition of $10^{-3}$ *M* quinidine to sperm cells preincubated in propranolol, the response appears to exceed by far that of a simple addition of the individual response rates.

## DISCUSSION

Membrane-stabilizing agents (local anesthetics and antiarrhythmic drugs) displace calcium from plasma membranes. Perturbation of the calcium not only affects cell permeability (Blaustein and Goldman, 1966) but the drugs, as ligands for $Ca^{2+}$-binding sites, also increase contractile tension in muscle (Bondani and Karler, 1970). Displacement of the calcium required for biological processes may enhance or disrupt flagellar activity.

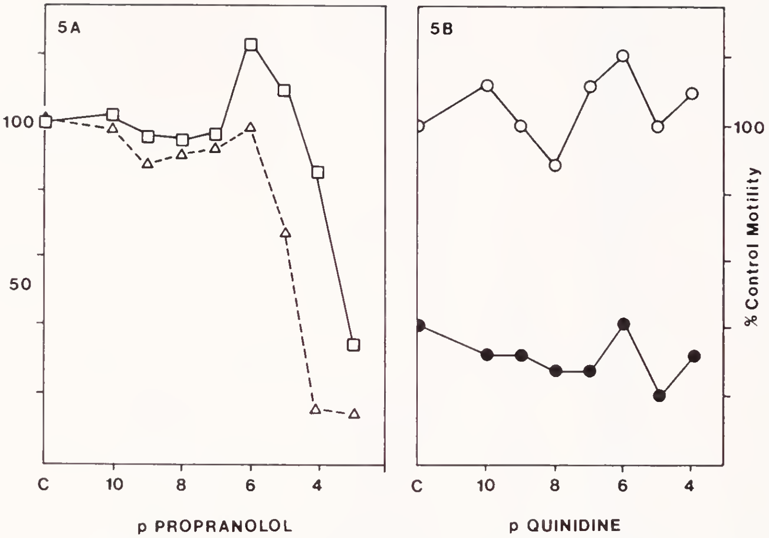

FIGURE 5. Separate effects of varying concentrations of propranolol (A) and quinidine (B) on *Arbacia* sperm in filtered sea water. 5A, triangles, varying concentrations of propranolol without preincubation; square, effects of preincubating in propranolol for 10 min prior to rating motility. 5B, open circles, varying concentrations of quinidine without preincubation; closed circles, same preparation 10 min later, showing that quinidine does not alter the effect of dilution in FSW. Note that while higher concentrations of propranolol markedly depress motile rate, quinidine's effects are fairly constant over the range of concentrations from 0.1 n*M* to 1.0 m*M*. Ordinates, relative motility in percent of control rate; abscissas, drug concentrations in negative log of molar concentration.

Brief exposure to procaine greatly increased *Arbacia* sperm forward motility; however, prolonging the incubation at the same concentration in filtered sea water led to complete cessation of progressive movement (Nelson, 1972). Moreover, EDTA sharply depressed the swimming rate of sea urchin sperm (Young and Nelson, 1974a). The calcium-selective chelator, EGTA, acted similarly at somewhat lower concentrations and sharpened the focus on a critical role for $Ca^{2+}$ (Young and Nelson, 1974b).

Procaine's action was thought to reflect an initial transitory increase in the internal free calcium released from sequestration sites in the membrane; whereas, blockage of the cell's ability to restore calcium to its resting distribution would account for the delayed inhibitory response (Nelson, 1972). These conclusions have been supported by the acceleration and prolongation of motility both in artificial sea water relatively low in heavy metal contaminants, and following procaine treatment in the MBL-ASW. The motility enhancement in MBL-ASW occurs in contrast to the loss of propulsive ability when the spermatozoa are preincubated in FSW solutions of procaine. The immediate response of the sperm cells to procaine in natural sea water resembles the responses observed in synthetic sea water solutions containing only minuscule amounts of heavy metals. If local anesthetics displace calcium from its binding sites in the plasma membrane, then, in the case of sea urchin sperm, at least part of that $Ca^{2+}$ which was released into the cell interior thereby increasing contractile activity was subsequently unable to be restored to physiological levels. In the synthetic salt medium the depressant effects of an excess of intracellular free calcium may be partially alleviated since $Ca^{2+}$ binding sites on the outer surface of the plasma membrane are not occupied by heavy metal ligands.

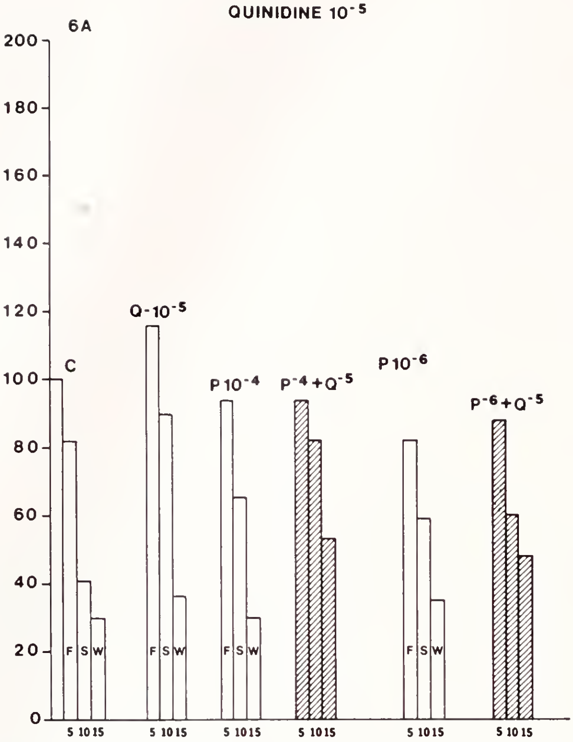

FIGURE 6. Interactive effects of quinidine (Q) and propranolol (P) on progressive movement of *Arbacia* sperm in FSW. Fig. 6A: In all panels, sperm cells were preincubated in FSW for 5 min to establish control rate (100%). At 5 min, 0.2 ml of FSW was added to the sperm suspensions in panel C, Q-$10^{-5}$, P-$10^{-4}$ and P-$10^{-6}$; 10 $\mu M$ quinidine was present in panels Q-$10^{-5}$, $P^{-4}$ + $Q^{-5}$ and $P^{-6}$ + $Q^{-5}$; to panels marked P-$10^{-4}$ and P-$10^{-6}$ propranolol was added to those final molar concentrations. Open bars contained only the one drug indicated (quinidine or propranolol); hatched bars represent cuvettes containing the drug mixtures. Motility ratings were made at three 5-minute intervals. Note that in this series no significant changes in the motile rate were caused by the drugs. Fig. 6B: Similar conditions to those depicted in 6A, except that 1 m$M$ quinidine was tested instead of 10 $\mu M$. In all panels, the sperm cells were preincubated for 5 min in FSW. Then 0.2 ml of FSW was added to the sperm suspensions in the cuvettes represented by the open bars. To the hatched-bar cuvettes was added 0.2 ml of propranolol at a final concentration of $10^{-4}$ $M$ and $10^{-6}$ $M$ respectively. Panels Q-$10^{-3}$, $P^{-4}$ + $Q^{-3}$ and $P^{-6}$ + $Q^{-3}$ contained quinidine, 1 m$M$. Cuvettes represented by open-bar panels contained only quinidine or propranolol alone as indicated. Motility was again rated at 5 min intervals. Note in this series that propranolol alone or in combination with quinidine increased the progressive motion over both the rates of the control and quinidine-alone panels, while in combination, $10^{-3}$ $M$ quinidine plus $10^{-6}$ $M$ or $10^{-4}$ $M$ propranolol, a marked increase in sperm speed occurred.

McGrady (1979) reported that $10^{-3}$ $M$ ouabain significantly depressed the membrane potential of bull sperm and at the same time caused decreases in the frequency and amplitude of the flagellar wave as well as in progressive movement of the cells.

Potentiation of these effects by $10^{-9}$ $M$ ouabain in MBL-ASW suggests that a fine and sensitive balance in the ultimate partition of the calcium across the cell membrane and within the cytoplasmic components must be maintained physiologically and that the presence of heavy metal ions in the environment disrupts the physiological balance.

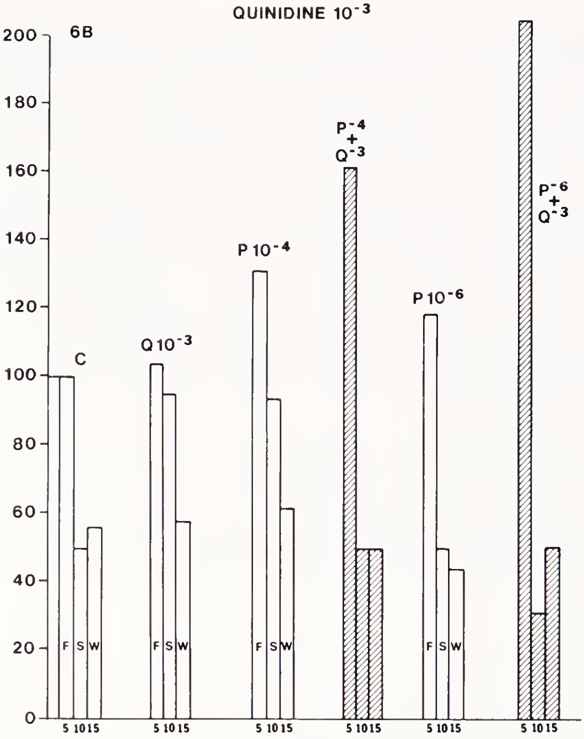

FIGURE 6 (*Continued*)

Ouabain does not compete for $Ca^{2+}$ binding sites, but the cardiac glycoside appears to act on a system involved directly or indirectly in transmembrane $Ca^{2+}$ extrusion. Electron micrographs show cytochemically that, in Ca-loaded, ejaculated bull sperm, ouabain causes the calcium to accumulate at the inner surface of the plasma membrane (Nelson *et al.*, 1980, 1982) as would be predicted on the basis of inactivation of a membrane-sited calcium-extrusion pump. Propranolol antagonizes the effect of ouabain; the degree of interaction depends on the relative concentrations of the drugs. The responses to the two drugs may be ascribed to the differences in sites and modes of their action as indicated above. Both propranolol and quinidine are cardiac antiarrhythmics, although propranolol is a beta-adrenergic receptor blocker and quinidine acts as a blocker of alpha-adrenergic receptors. Quinidine (Fig. 5b) alone caused relatively little change in motility from that of the untreated controls. When $10^{-5}$ *M* quinidine alone was tested there was a 40% increase over the control motility in FSW (Fig. 6a), and when tested after the addition of propranolol, motility remains essentially unaffected. However, when $10^{-4}$ *M* or $10^{-6}$ *M* propranolol (final concentration) was added to the suspensions preincubated in $10^{-3}$ *M* quinidine in FSW, the motility during the first five minutes shot up to 160% and 200% of the control levels, respectively, before dropping back down to the same level as that of the controls during prolongation of the incubation periods.

Rothschild and Tyler (1954) suggested that sperm cells incubated in the chemically defined synthetic medium exhibit greater activity for longer periods by not being exposed to heavy metal contaminants found in natural sea water. However, the chelating agents EDTA and EGTA exerted only depressant effects on *Arbacia* sperm motility in FSW at all concentrations assayed (Young and Nelson, 1974b). Interference with any of a number of Ca-dependent processes could lead to aberrant behavior. Calcium entry may be restricted by omission or removal (*e.g.* by EGTA) of calcium from the cells' environment. Binding sites on the cell surface may be occupied reversibly or irreversibly by competitive ligands ($La^{3+}$, $Cu^{2+}$, $Pb^{2+}$, $Ni^{2+}$); entry channels may be impeded or inactivated (chelators, $La^{3+}$, anticholinergic agents); calcium may be displaced from sites within the plasma membrane ("membrane-stabilizer"); binding sites on $Ca^{2+}$-dependent enzymes may be occupied by other cations ($Mn^{2+}$, $Zn^{2+}$); calcium extrusion may be inhibited (ouabain or inhibition of enzymes responsible for ATP synthesis). Sparing ATP by inhibition of other ATP-utilizing systems also increases sperm motility.

The time- and dose-dependence of the responses to the transmembrane differential distribution of calcium, as well as to the displacement of calcium ions from binding sites, appear to operate in the case of the sperm cell membrane as in other excitation-effector systems. The plasma membrane is endowed with a variety of receptors with some sites showing affinity for $Ca^{2+}$ which is subject to displacement by membrane-soluble agents; such a membrane exhibits selective ionic permeability and an environmentally sensitive membrane potential.

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# TWO CELL VOLUME REGULATORY SYSTEMS IN THE *LIMULUS* MYOCARDIUM: AN INTERACTION OF IONS AND QUATERNARY AMMONIUM COMPOUNDS

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## ABSTRACT

The horseshoe crab *Limulus polyphemus* is extremely euryhaline. Previous studies have shown it surviving in salinities ranging from 6% to 200% sea water. Blood osmotic concentration is hyperregulated in low salinities, but above 65% sea water *Limulus* is an osmoconformer. *Limulus* regulates cell volume when exposed to low salinity, despite a small intracellular free amino acid pool. Instead, the quaternary ammonium compound glycine betaine is the major nitrogenous osmotic solute in *Limulus* heart tissue. However, volume regulation is complete before intracellular glycine betaine concentrations change. Isolated heart tissue exposed to low salinity shows no change in glycine betaine levels in 24 h though volume regulation occurs. During the initial phase of volume regulation intracellular $Na^+$ and $Cl^-$ content in the isolated tissue decreases markedly with exposure to low salinity. Therefore, *Limulus* utilizes two osmotic solute types during cell volume regulation: $Na^+$ and $Cl^-$ initially and glycine betaine later.

## INTRODUCTION

The utilization of free amino acids as intracellular osmotic solute to regulate cell volume in euryhaline invertebrates exposed to external osmotic stress has been considered ubiquitous (Gilles, 1979; Pierce and Amende, 1981). Free amino acids often make up more than 50% of the intracellular osmotic solute in marine invertebrates (Gilles, 1979). The levels of these intracellular free amino acids are adjusted in response to changes in extracellular osmotic concentration, controlling the volume of water in the cells. In contrast to other euryhaline invertebrates, the tissues of the horseshoe crab, *Limulus polyphemus*, contain only low levels of free amino acids, making up 10% or less of the osmotically active substances (Bricteux-Gregoire *et al.*, 1966; Prior and Pierce, 1981) despite its wide salinity tolerance. *Limulus* has been found living in salinities ranging from 7 to 30 ppt (McManus, 1969) and survived in experimental salinities of 3 to 64 ppt (Robertson, 1970). Furthermore, the free amino acid concentration in *Limulus* tissue drops only slightly with acclimation to low salinity (Prior and Pierce, 1981).

Although *Limulus* cells do not utilize amino acids as a main osmotic solute, the total non-protein nitrogen content of *Limulus* tissue is substantial and changes considerably with external salinity (Bricteux-Gregoire *et al.*, 1966; Robertson, 1970). The identity of this nitrogenous solute is unknown, but there are some obvious possibilities. In particular, quaternary ammonium compounds are common in in-

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Abbreviations: HPLC, high performance liquid chromatography; MOPS, morpholinopropanesulfonic acid; mosm, milliosmoles per kilogram water; TCA, trichloroacetic acid; TLC, thin layer chromatography.

vertebrate tissues (Welsh and Prock, 1958; Gasteiger *et al.*, 1960; Beers, 1967; Robertson, 1980) and, where it has been tested, these compounds vary with salinity (Bricteux-Gregoire *et al.*, 1962, 1964; Dall, 1971; Norton and de Rome, 1980) but usually constitute only a minor part of the nitrogenous osmotic solute pool. Quaternary ammonium compounds are present in *Limulus* (Ackermann and List, 1958) and might account for at least part of the unidentified pool of osmotic solute in *Limulus*, although Levy (1967) was unable to demonstrate such a relationship.

Another possible pool of intracellular osmotic solute is inorganic ions, although the evidence indicating the use of these substances by osmotically stressed invertebrate cells is limited. However, inorganic ions play a role in volume regulation in the few invertebrate species studied, both as an initial source of solute (Kevers *et al.*, 1981) and throughout the acclimation period (Freel, 1978; Willmer, 1978). $K^+$, $Na^+$, and $Cl^-$ have all been implicated in some combination (Freel, 1978; Kevers *et al.*, 1979; Treherne, 1980).

Because of its extreme euryhalinity and small amino acid pool, we have investigated the possible role of these alternate solutes in the cell volume regulation of *Limulus* heart tissue. The results show that the quaternary ammonium compound glycine betaine is the major nitrogenous osmotic solute in heart tissue taken from crabs acclimated to low salinities. However, isolated heart tissue exposed to low salinity shows no decrease in glycine betaine or $K^+$ content in 24 h, but rather a large decrease in $Na^+$ and $Cl^-$ content. Therefore, *Limulus* utilizes both organic compounds and inorganic ions to regulate cell volume during low salinity stress. A preliminary report of these findings has appeared previously (Warren and Pierce, 1981).

## MATERIALS AND METHODS

### Limulus *acclimated to low salinity*

*Limulus*, obtained from The Marine Biological Laboratory, Woods Hole, MA, were acclimated to salinities ranging from 930 mosm to 55 mosm, at 14–18°C, for 2–3 weeks. Natural sea water, diluted appropriately with deionized water, was used. After the acclimation period, blood and tissue samples were taken to determine the low-salinity acclimated levels of blood osmotic concentration, tissue amino acids, total non-protein nitrogen and quaternary ammonium compounds, as indicated below.

Blood was withdrawn from the acclimated animals by insertion of a syringe into the pericardial sinus at the joint between the prosoma and the opisthosoma. The blood was centrifuged at $20{,}000 \times g$ to remove cells and clots, and the osmotic concentration of the supernatant determined using a freezing point depression osmometer (Precision Systems Osmette).

The heart was exposed by cutting away a dorsal section of the carapace. A small section of the cardiac tissue was removed, blotted, and weighed. The tissue was then lyophilized and reweighed to determine tissue hydration, as percent wet weight lost by lyophilization.

Intracellular amino acids were extracted from the dried tissue samples by homogenization in 40% ethanol, followed by boiling and centrifugation to remove protein. The supernatant was lyophilized and the residue resuspended in lithium citrate buffer (pH 2.2). The amino acid composition of this solution was determined with an amino acid analyzer (JOEL JLC-6AH).

Tissue non-protein nitrogen concentrations were also determined. Lyophilized heart tissue was homogenized in ice cold distilled water. Ice cold trichloroacetic acid

(TCA) was added to the homogenate to give a final concentration of 10% TCA. The precipitated protein was spun down at 20,000 × *g* and the supernatant frozen until analysis. A portion of each sample was added to tubes containing 0.1 g digestion mixture ("Sel-dahl" copper-selenite mixture, Scientific Products), followed by 0.5 ml of concentrated $H_2SO_4$. The tubes were heated to 320°C and the samples digested at that temperature for 2 h to break down nitrogenous compounds to ammonia (Lang, 1958). After cooling, the samples were diluted with distilled water and a portion from each was placed into a glass vial and neutralized with 50% KOH. The vials were quickly capped with rubber stoppers, each of which held a glass rod with a ground tip extending into the vial. Prior to insertion into the vial, a drop of 1 *N* $H_2SO_4$ was applied to the tip of each glass rod (Seligson and Seligson, 1951). The vials were rotated at an angle overnight. The ammonia released from the basic solution was trapped in the acid on the glass rods, forming ammonium sulfate. This was rinsed off with distilled water, and the ammonia content determined colorimetrically (Liddicoat *et al.*, 1975). Both ammonium sulfate and glycine betaine were run through the entire procedure as standards.

Intracellular quaternary ammonium compounds were measured in *Limulus* cardiac tissue by reineckate precipitation (Barnes and Blackstock, 1974). Tissue samples were extracted as described above for amino acid analysis, but the lyophilized supernatants were resuspended in distilled water rather than buffer. A portion of this solution from each tissue sample was applied to mixed bed ion-exchange columns (Dowex-1 and Amberlite-50, 2:1) and washed with water, removing any interferring amino acids. Then 1 *N* HCl was added and mixed with the washings, followed by saturated, filtered ammonium reineckate (pH 1). The quaternary ammonium compounds precipitated while standing overnight at 4°C. The precipitates were then filtered from each solution, using polycarbonate membrane filters (Bio-Rad, 0.2 μm) in syringe filter holders (Millipore). Once an entire sample was filtered, excess reineckate was removed by passing ether across the filter several times. The filter was removed, the precipitate dissolved in 70% acetone-water, and the absorbance read at 520 nm. Glycine betaine standards were run with each group of samples.

Preliminary investigations using standard paper and thin layer chromatography (TLC) (Bregoff *et al.*, 1953; Hayashi and Konosu, 1977) confirmed that the quaternary ammonium compounds glycine betaine and homarine were present in *Limulus* heart tissue. Concentrations of glycine betaine and homarine were then measured directly using high performance liquid chromatography (HPLC) (Altex). A reverse phase column (Spherisorb C-6, 5 μm particle size, Chromanetics) and a mobile phase of 0.1 *M* phosphate buffer (pH 3), containing 1 m*M* octane sulfonic acid as an ion-pairing reagent, were used to separate the compounds. Lyophilized samples were resuspended in water and a portion of each, appropriately diluted with mobile phase buffer, was injected onto the column. A UV monitor (Gilson) detected the compounds at 190 nm, and concentration was determined with a data processor (Shimadzu). Identification of peaks was verified by spiking samples with standards to obtain a single, larger peak and by collecting the column eluent containing each peak and running them on TLC.

### *Time course of acclimation*

To determine the time course of events occurring during the low salinity acclimation process, changes in blood osmotic concentration, and cardiac tissue and blood glycine betaine levels were followed from the time of transfer of crabs to low salinity to the end of the two-week acclimation period. Prior to the experimental period, animals were maintained at 10°C in artificial sea water (Instant Ocean, 930 mosm). Animals were then transferred from 930 mosm to 235 mosm; control animals were kept at 930 mosm.

Cardiac tissue samples were taken from both groups at various intervals throughout the two-week acclimation period. Glycine betaine in these tissues was measured as described above. Blood samples were also withdrawn from the animals in both groups as previously described. After centrifugation, the osmotic concentration of a portion of each blood sample was determined. The remainder of the blood samples were deproteinized by addition of appropriate amounts of ethanol to a final concentration of 40%, brought to a boil, and then centrifuged to remove the precipitate. The supernatant was analyzed for glycine betaine by HPLC.

### *Isolated tissue response to low salinity*

The response of isolated *Limulus* hearts to low salinity stress was also investigated by measuring changes in tissue hydration, glycine betaine and intracellular ions. In order to demonstrate cell volume regulatory ability in isolated hearts, initial studies measured weight changes in isolated hearts exposed to low salinity. Hearts were dissected from *Limulus* acclimated to 930 mosm artificial sea water (10°C) and placed in saline (940 mosm, 10°C, ionic content in Table I). The hearts were carefully cleaned of any tissue debris and rinsed several times with fresh saline. Hearts were then put into either 940 mosm or 400 mosm saline and maintained at 10°C. (The 400 mosm saline is approximately the same osmotic concentration as blood taken from animals acclimated to 235 mosm.) The hearts were removed from the saline, blotted, and weighed at intervals up to 12 or 24 h. Changes in weight were expressed as percent initial wet weight for each heart.

To measure intracellular ions and glycine betaine, the dissected hearts were cleaned and split longitudinally along the ventral side and then cut into two sections across the width. One section of each heart was transferred to 400 mosm saline, while the other half was transferred to 940 mosm saline, both solutions containing $^{14}$C-polyethylene glycol (MW 4000, New England Nuclear) as an extracellular marker (4 h required for complete equilibration in the extracellular space). The tissue pieces were maintained at 10°C with aeration and light shaking. Media and tissue were sampled at 6 and 12 h intervals. Each tissue sample at each interval was

TABLE I

*Ionic concentrations of saline used in isolated* Limulus *heart experiments.*

| | m*M* |
|---|---|
| NaCl | 420 |
| $MgCl_2$ | 30 |
| $MgSO_4$ | 20 |
| KCl | 11 |
| $CaCl_2$ | 11 |
| $NaHCO_3$ | 5 |
| MOPS | 5 |
| mosm | 940 |
| pH | 7.5 |

Lower salinities were made by dilution with distilled water, but maintaining the MOPS concentration.

divided into three pieces. Each piece was blotted and weighed. One was lyophilized and reweighed to determine tissue hydration, and then used for measurement of glycine betaine, as described previously. The ions were extracted from a second tissue piece with 1 *N* nitric acid. The third piece was solubilized in Protosol (New England Nuclear) and the $^{14}C$-polyethylene glycol content measured by liquid scintillation counting. Radioactivity in a sample of the media was also determined, and ratios of these two counts were used to determine extracellular space.

$Na^+$ and $K^+$ concentrations were measured with an atomic absorption spectrophotometer (Perkin-Elmer Model 560). Samples of the tissue extracts and the incubation media were diluted with a solution containing 1% nitric acid and an excess of $K^+$ or $Na^+$ as applicable to prevent ionization. Standards also contained $Ca^{++}$ and $Mg^{++}$ in concentrations proportional to sea water. Chloride was measured in samples of extracting fluid and media using a chloridometer (Buchler-Cotlove).

### *Intracellular ion concentrations in acclimated animals*

Intracellular ion concentrations were measured in hearts taken from animals acclimated to low salinity for 14 to 16 days. *Limulus* were acclimated to either 930 mosm or 235 mosm at 10°C. The acclimation salinity of 235 mosm results in blood osmotic concentrations of approximately 400 mosm, the osmotic concentration used for the isolated tissue experiments.

After the acclimation period, blood was collected from the animals and centrifuged as previously described. The hearts were then removed and quickly cleaned without rinsing in saline. Two pieces of tissue were excised from each heart and quickly processed: one was blotted, weighed, and lyophilized for determination of tissue hydration; the other was blotted, weighed, and placed in 1 *N* nitric acid for ion extraction. $Na^+$, $Cl^-$, and $K^+$ were measured as previously described. The remainder of each heart was incubated in blood, collected from the animal, to which $^{14}C$-polyethylene glycol was added. The tissue sections were maintained with aeration and shaking for 4 h, to allow equilibration of the polyethylene glycol in the extracellular space. A piece of the heart was then removed, blotted, and weighed. The tissue and a portion of the blood were solubilized in Protosol and radioactivity determined by liquid scintillation counting. All intracellular ion concentrations were calculated with correction for the extracellular space (Freel *et al.*, 1973).

### *Statistical analysis*

Statistical significance was determined by analysis of variance and Student's *t* test. A probability of $P < 0.02$ was considered significant. All data are expressed as means ± S.E.

## RESULTS

### Limulus *acclimated to low salinities*

In salinities from 700 mosm to 930 mosm, the blood osmotic concentration of *Limulus* varies directly with that of the external medium. Over this salinity range, the blood is slightly hyperosmotic to the medium (16 to 27 mosm) (Fig. 1). In the more dilute salinities, from 55 mosm to 600 mosm, the blood osmotic concentration is maintained well above that of the medium (52 to 307 mosm) as both Robertson (1970) and Mangum *et al.* (1976) reported.

Tissue hydration remained constant over the entire salinity range tested (Fig. 2). Analysis of variance revealed no significant differences in treatment means.

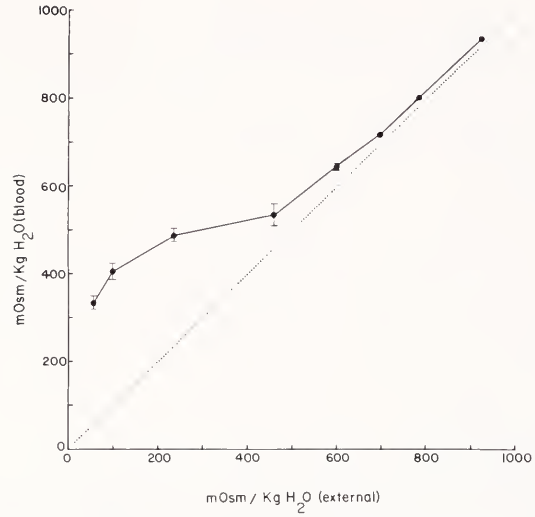

FIGURE 1. Blood osmotic concentration of *Limulus* acclimated to a range of salinities.

The total size of the free amino acid pool in heart tissue taken from *Limulus* acclimated to full strength sea water (930 mosm) is 170 μmoles/g dry weight. The pool size generally decreases with acclimation to lower salinities (Fig. 3). The major amino acid in *Limulus* heart tissue is taurine (Table II), making up nearly 50% of the total pool at 930 mosm and decreasing with salinity, especially at the lower acclimation salinities. In contrast, the total non-protein nitrogen in cardiac tissue is nearly 1300 μmoles/g dry weight and shows substantial decrease with acclimation to low salinity (Fig. 4).

A large portion of the non-protein nitrogen is accounted for by quaternary ammonium compounds, which are 750 μmoles/g dry weight in hearts of animals acclimated to 930 mosm (Fig. 4). Furthermore, the quaternary ammonium compound concentration decreases with acclimation to 700 mosm and 460 mosm salinities. Glycine betaine and homarine account for most of the quaternary ammonium compound pool (514 and 139 μmoles/g dry weight, respectively) at 930 mosm acclimation salinity (Fig. 5). Glycine betaine concentration decreases substantially over the range of acclimation salinities, whereas homarine shows only a slight decrease (Fig. 5).

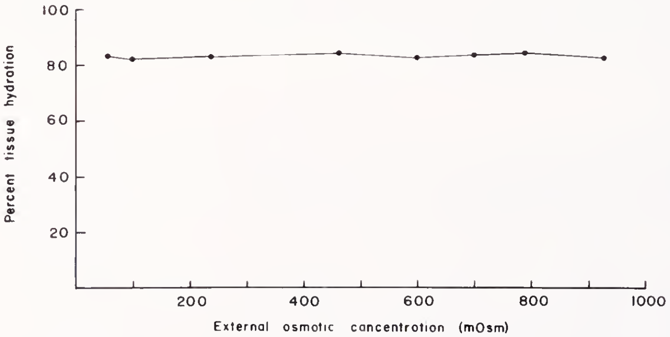

FIGURE 2. Percent hydration of heart tissue taken from *Limulus* acclimated to a range of salinities. Standard errors are smaller than the size of the points.

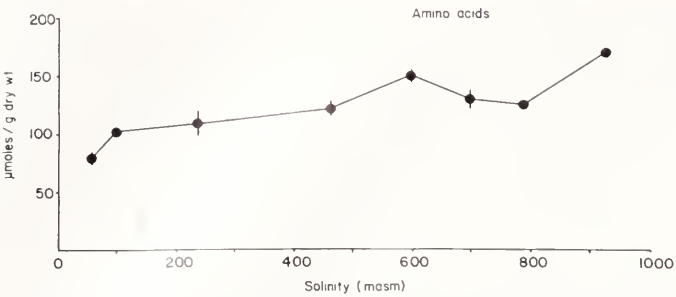

FIGURE 3. Total amino acid pool in heart tissue taken from *Limulus* acclimiated to a range of salinities.

### *Time course of acclimation*

Following transfer directly from an external salinity of 930 mosm to 235 mosm, the blood osmotic concentration of *Limulus* drops rapidly to within 100 mosm of the final blood osmotic concentration during the first 24 h, and levels off within 48 h (Fig. 6 top). However, tissue glycine betaine does not decrease substantially until 48 h, and then only slowly declines up to day 7 (Fig. 6 middle). Blood glycine betaine concentrations reflect the tissue changes, not reaching a peak until 48 to 72 h and then gradually declining through day 14 (Fig. 6 bottom).

### *Isolated tissue response to low salinity*

Isolated *Limulus* hearts taken from animals acclimated to 930 mosm and then exposed directly to 400 mosm saline gain 140% of initial wet weight in 2 h (Fig. 7). The weight then decreases to 123% of original by 12 h and finally recovers back to 117% of initial weight by 24 h.

In spite of this volume regulation, no significant change in tissue glycine betaine concentrations occurred after 6, 12, and 24 h of incubation in low salinity (Table III). However, significant decreases in inorganic ion concentrations occurred at these sampling intervals. Intracellular $K^+$ concentration decreases slightly in the tissues exposed to low salinity, but no more than can be accounted for by cell swelling (Table IV). The intracellular $K^+$ content (mmoles/kg dry weight) does not decrease in low salinity. Intracellular $Na^+$ and $Cl^-$ concentrations decrease drastically during exposure of the isolated heart tissues to 400 mosm saline (Table V and VI). This decrease is significantly lower than that predicted by cellular hydration changes, and the $Na^+$ and $Cl^-$ contents (mmoles/kg dry weight) also decrease significantly in these tissues exposed to low salinity.

TABLE II

*Major amino acids (μmoles/g dry wt ± S.E.) in heart tissue taken from* Limulus *acclimated to full-strength sea water (930 mosm).*

| | |
|---|---|
| Tau | 79.5 ± 5.7 |
| Glu | 24.9 ± 2.8 |
| Pro | 23.7 ± 3.3 |
| Arg | 21.7 ± 1.8 |
| Ala | 7.9 ± 1.1 |
| Orn | 4.8 ± 1.5 |
| Asp | 3.6 ± 0.8 |

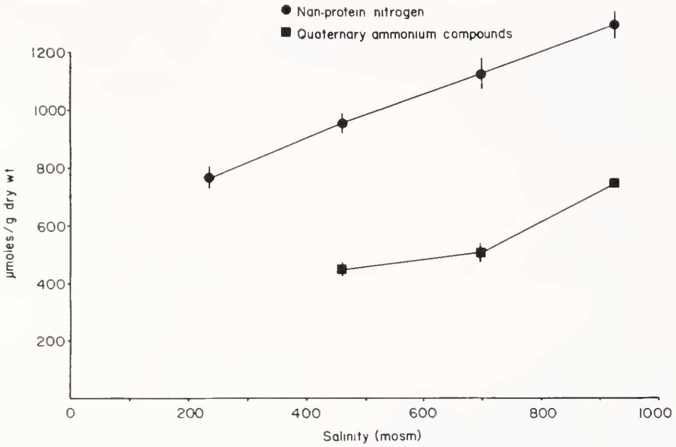

FIGURE 4. Non-protein nitrogen and quaternary ammonium compounds in heart tissue taken from *Limulus* acclimated to the salinities shown.

### *Ion concentrations in acclimated animals*

The levels of intracellular inorganic ions in heart tissue taken from animals acclimated to low salinity are different from those in the isolated heart tissue following exposure to low salinity (Table VII). Intracellular $Na^+$ and $Cl^-$ levels in the low salinity acclimated animals are significantly lower than in the high salinity acclimated animals. However, the $Na^+$ and $Cl^-$ levels in the low salinity acclimated animals are significantly increased from the levels in the isolated tissue after a 12 h exposure to low salinity. Furthermore, intracellular $K^+$ in the low salinity acclimated animals is significantly decreased from the levels in high salinity acclimated animals, even though $K^+$ content in the isolated tissues did not decrease.

## DISCUSSION

The extreme euryhalinity of *Limulus* can be accounted for by two general physiological processes. First, we found as did Robertson (1970) that *Limulus* is an

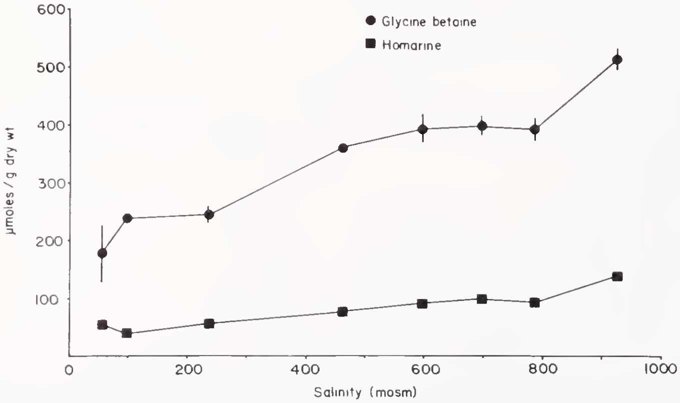

FIGURE 5. Glycine betaine and homarine concentrations, measured by HPLC, in heart tissue taken from *Limulus* acclimated to a range of salinities.

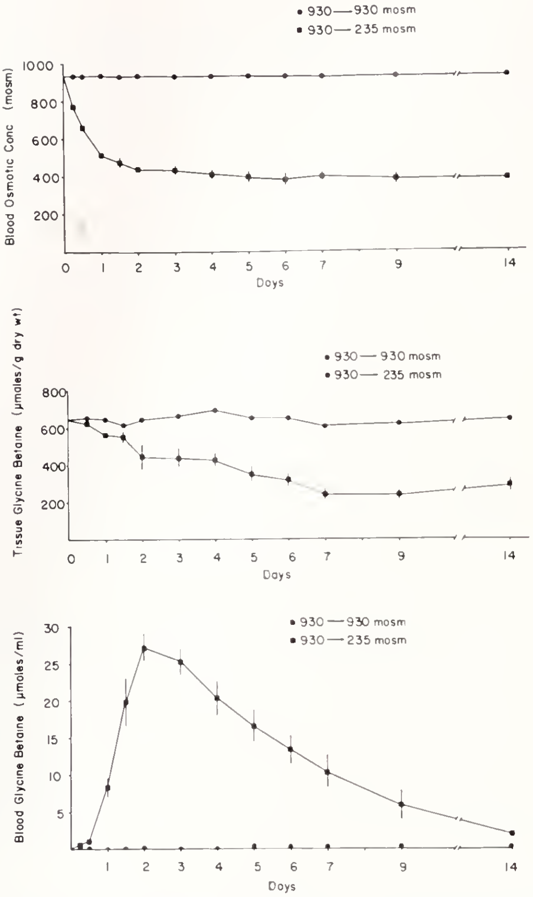

FIGURE 6. Time course of changes in blood osmotic concentration (top), tissue glycine betaine (middle) and blood glycine betaine (bottom) of *Limulus* acclimated to 930 mosm and exposed to 235 mosm. Values for control animals kept in 930 mosm are also shown.

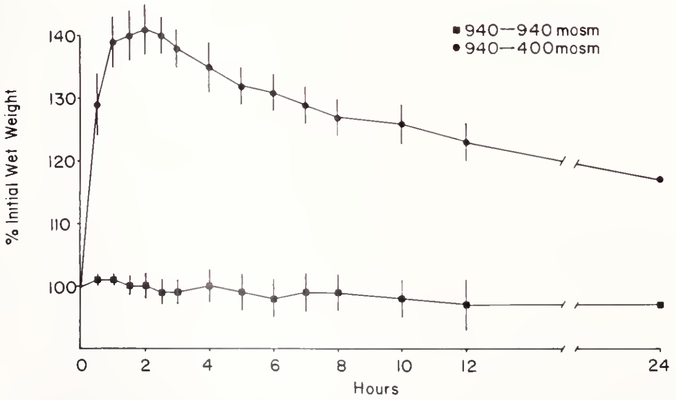

FIGURE 7. Time course of changes in wet weight, as % initial wet weight, of isolated hearts, taken from *Limulus* acclimated to 930 mosm, and exposed to 400 mosm or 940 mosm.

TABLE III

*Glycine betaine (μmoles/g dry wt ± S.E.) in isolated heart tissue from* Limulus *acclimated to 930 mosm.*

| | 940 mosm | 400 mosm |
|---|---|---|
| 6 h | 599 ± 24 | 633 ± 15 |
| 12 h | 621 ± 16 | 631 ± 27 |
| 24 h | 585 ± 21 | 620 ± 21 |

The low salinity values are not significantly different from the high salinity controls.

osmoregulator in salinities below 600 mosm. Second, *Limulus* has a substantial ability to regulate cell volume. The basis of this cellular mechanism is the utilization of two types of intracellular osmotic solutes: small molecular weight nitrogenous compounds and inorganic ions. Unlike many invertebrates, *Limulus* has only a small intracellular free amino acid pool. Instead, the quaternary ammonium compound glycine betaine is the major nitrogenous osmotic solute in *Limulus* heart tissue. This compound is a common constituent in many invertebrates, but usually in small amounts (Robertson, 1961, 1965, 1980; Beers, 1967). Glycine betaine occurs in substantial amounts in some molluscs (*Mytilus*, Bricteux-Gregoire *et al.*, 1964; *Tapes*, Norton and de Rome, 1980), in association with substantial amino acid pools.

Free amino acid concentrations in the cells of intact euryhaline invertebrates normally fall rapidly during low salinity stress, often reaching the final lowered concentration within a day or two (Dall, 1975; Bartberger and Pierce, 1976). In contrast, glycine betaine concentrations slowly decreased over 7 days in the heart tissues of *Limulus* acclimating to low salinity, long after the drop in blood osmotic concentration occurred. However, the 100-fold increase of blood glycine betaine concentrations during the period of glycine betaine decrease in the tissues indicates that glycine betaine is effluxed intact from the cells, in a manner similar to free amino acid utilization by other species (Pierce and Amende, 1981). Thus, glycine betaine is only slowly utilized as osmotic solute and not at all in the initial stages of salinity acclimation in *Limulus* heart tissue. This is confirmed by our isolated tissue experiments.

The isolated *Limulus* heart volume regulates during exposure to hypoosmotic media. The pattern of volume regulation by this tissue is typical of that found in

TABLE IV

*Intracellular $K^+$ in isolated heart tissue from* Limulus *acclimated to 930 mosm.*

| | mmoles/kg $H_2O$ | | | mmoles/kg dry wt | |
|---|---|---|---|---|---|
| Salinity | 940 mosm | 400 mosm | Predicted* | 940 mosm | 400 mosm |
| 6 h | 112.6 ± 7.5 | 74.7 ± 2.9 | 75.0 ± 5.2 | 458 ± 29 | 432 ± 14 |
| 12 h | 113.0 ± 5.7 | 83.2 ± 3.2 | 73.6 ± 4.3 | 432 ± 28 | 453 ± 11 |
| 24 h | 114.5 ± 5.3 | 88.6 ± 3.5 | 79.3 ± 4.6 | 458 ± 20 | 511 ± 13 |

* Calculated according to Freel *et al.* (1973).

The data are expressed two ways. $K^+$ concentration (mmoles/kg $H_2O$) decreases during low salinity exposure but only as much as predicted by changes in tissue hydration. There is no significant decrease in $K^+$ content (mmoles/kg dry wt), indicating that $K^+$ is not used as osmotic solute.

TABLE V

*Intracellular $Na^+$ in isolated heart tissue from* Limulus *acclimated to 930 mosm.*

| | mmoles/kg $H_2O$ | | | mmoles/kg dry wt | |
|---|---|---|---|---|---|
| Salinity | 940 mosm | 400 mosm | Predicted* | 940 mosm | 400 mosm |
| 6 h | 237.7 ± 13.0 | 79.0 ± 16.9 | 153.1 ± 8.9 | 913 ± 51 | 437 ± 89 |
| 12 h | 228.9 ± 17.6 | 46.3 ± 6.3 | 144.5 ± 10.6 | 905 ± 72 | 273 ± 34 |

* Calculated according to Freel *et al.* (1973).

$Na^+$ concentration (mmoles/kg $H_2O$) decreases substantially during low salinity exposure, significantly more than predicted by hydration changes. $Na^+$ content (mmoles/kg dry wt) also shows a very significant decrease during low salinity stress, indicating that $Na^+$ is used as osmotic solute.

other cell types: a rapid swelling followed by an incomplete recovery (reviewed by Gilles, 1979). Cellular volume regulation in response to hypoosmotic stress is achieved by a reduction in the amount of intracellular organic osmotic solute. In most invertebrate cells the solute reduction is accomplished by a rapid efflux of amino acids, but in the isolated *Limulus* heart, glycine betaine levels remained constant throughout the 24 h exposure to low salinity even though volume regulation was occurring. Thus, the initial control of cell volume in the *Limulus* tissue must rely on an alternate solute source. Our results indicate that intracellular $Na^+$ and $Cl^-$ provide that function. Intracellular $Na^+$ and $Cl^-$ contents decrease in isolated heart tissue exposed to low salinity, and the decrease occurs quickly, within the first 6 h. Therefore, the isolated heart volume regulates utilizing the high intracellular $Na^+$ and $Cl^-$ contents as osmotic solute, without any changes in the level of glycine betaine.

The utilization of $Na^+$ and $Cl^-$ as initial osmotic solute explains the lag time between the decline in blood osmotic concentration and changes in glycine betaine in the cells of the acclimating whole animal. $Na^+$ and $Cl^-$ probably serve as the initial osmotic solute during the first day or two of exposure of the whole animal to low salinity, with the glycine betaine utilization occurring slowly as the first week of acclimation proceeds. In part, glycine betaine replaces $Na^+$ and $Cl^-$ as osmotic solute during the acclimation process, shown by the partial return of $Na^+$ and $Cl^-$ levels towards original.

TABLE VI

*Intracellular $Cl^-$ in isolated heart tissue from* Limulus *acclimated to 930 mosm.*

| | mmoles/kg $H_2O$ | | | mmoles/kg dry wt | |
|---|---|---|---|---|---|
| Salinity | 940 mosm | 400 mosm | Predicted* | 940 mosm | 400 mosm |
| 6 h | 199.9 ± 16.0 | 60.3 ± 8.7 | 124.7 ± 9.4 | 780 ± 52 | 352 ± 56 |
| 12 h | 195.8 ± 13.5 | 39.5 ± 4.6 | 135.8 ± 12.8 | 762 ± 48 | 221 ± 26 |
| 24 h | 201.6 ± 15.3 | 38.8 ± 5.1 | 141.0 ± 8.3 | 834 ± 76 | 213 ± 24 |

* Calculated according to Freel *et al.* (1973).

$Cl^-$ concentration (mmoles/kg $H_2O$) decreases substantially during low salinity exposure, significantly more than predicted by hydration changes. $Cl^-$ content (mmoles/kg dry wt) also shows a very significant decrease during low salinity stress, indicating that $Cl^-$, like $Na^+$, is used as osmotic solute.

TABLE VII

*Intracellular ion content (mmoles/kg dry wt ± S.E.) of heart tissue taken from* Limulus *acclimated to 930 mosm or 235 mosm.*

| | Acclimated animals | | Isolated tissue* | |
|---|---|---|---|---|
| Salinity | 940 mosm | 235 mosm | 940 mosm | 400 mosm |
| $Na^+$ | 873 ± 47 | 401 ± 62 | 905 ± 72 | 273 ± 34 |
| $Cl^-$ | 853 ± 57 | 347 ± 55 | 762 ± 48 | 221 ± 26 |
| $K^+$ | 443 ± 16 | 361 ± 11 | 432 ± 28 | 453 ± 11 |

* Ion contents of isolated heart tissue after a 12 h exposure to 940 or 400 mosm are included for comparison.

Our results show that the utilization of glycine betaine as osmotic solute in *Limulus* heart tissue is very different from the mechanisms of free amino acid regulation in other euryhaline invertebrates. It is clear that *Limulus* cells utilize two very different types of osmotic solute. The solute control mechanisms are unknown and we are currently investigating them. However, our study indicates that the mechanisms controlling each of the solute levels are different, functioning with separate time courses. In spite of this difference, the mechanisms are coordinated so that cell volume is rapidly reduced by $Na^+$ and $Cl^-$ efflux, and the later glycine betaine efflux continues the acclimation process, maintaining and perhaps finely adjusting cell volume. Thus, there seem to be two permeability control systems acting in concert to regulate cell volume.

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